

MOLECULAR ANALYSIS OF GENETIC DIVERSITY IN FLORIDA SEA-OATS
(*UNIOLA PANICULATA*) POPULATIONS: NEW APPROACHES TO
GENERATE AND ANALYZE MOLECULAR DATA

By

DAYAWANSA G. RANAMUKHAARACHCHI

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This dissertation is dedicated to my beloved parents, my wife Nadika,
two sons, Sural and Sayuru and my brothers and sisters

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MOLECULAR ANALYSIS OF GENETIC DIVERSITY IN FOUR FLORIDA SEA-
OATS (*UNIOLA PANICULATA*) POPULATIONS: NEW APPROACHES TO
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By

Dayawansa G. Ranamukhaarachchi

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Chairman: Michael E. Kane

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Sea-oats (*Uniola paniculata* L.) is a pioneer coastal grass used for dune restoration projects. Concerns about altering the genetic constitution and population dynamics of sea-oats in restoration projects has generated a need to understand its inherent genetic variation and how that information might affect the successful development of restored dunes relative to their ecology and protection of coastal structures. To determine the genetic variation, sea-oats population studies were conducted using adult plants and seedlings from four Florida populations on the Atlantic and Gulf coasts. From each population, 23 adult plants and 18 seedlings were sampled. Random amplified polymorphic DNA (RAPD) generated data using 10 random primers were subjected to statistical analyses. Genetic analyses revealed that both adult and seedling plants displayed high within- and between- population genetic variation, and extensive genetic differentiation between the coasts. Highest within-population variation was

observed on the Gulf coast with St. George Island producing the highest variation, followed by Egmont Key. In contrast, the Atlantic populations showed the least within- and between- population differentiation. These population and coastal differences were attributed to: (1) frequent storm disruptions on the Gulf coast causing high genetic variation associated with population disturbances; (2) higher levels of gene flow between populations on the same coast favoring homogenization; and (3) restricted levels of gene flow between populations on the two coasts. Coastal genetic differences suggested possible ecotypic differences; however, common-garden and reciprocal transplant studies are required to assess actual adaptability.

New procedures to generate and analyze molecular data were developed. Genetic recombination, as revealed by molecular markers, was explored to obtain information on reproduction modes using adult sea-oats population genetic data. Based on this study, relative levels of genetic diversity, occurrence of clone-mates in populations, and spatial distribution and genetic relationships between clone-mates were found. A standard amplified fragment length polymorphism (AFLP) technique was modified to generate markers with high reproducibility and specificity, with the same convenience of RAPD. A comparison between modified AFLP technique and RAPD using a number of plant species showed that individuals, cultivars, and ecotypes can be efficiently and reliably differentiated using this modified AFLP technique.

CHAPTER 1 INTRODUCTION

Hurricanes and storms are a major source of physical disturbance in marine and coastal ecosystems. Both high winds and large waves cause damage leading to significant economic and ecological losses (Woodhouse, 1982). Coastal sand dunes serve as a natural defense system against these destructive forces (Crewz, 1987; Dahl and Woodard, 1977; Woodhouse, 1982). Degradation of coastal dune systems by natural forces or human activities has increased the risk of catastrophic ecological and economic losses from hurricanes. Fucella and Dolan (1996) reported a maximum loss of 70 cm sand depth in the middle of the sand profiles during a 12-hour tidal cycle during high storm action in North Carolina. Clark (1989), estimated that about 220 of Florida's 800 miles of sandy beaches were in a critical state of erosion. Continued protection of already eroded beaches and restoration of dunes are necessary to prevent additional losses. Constructing physical structures, renourishing beaches with sand pumped from offshore, and planting these areas with appropriate indigenous plant species are the more accepted practices for controlling coastal erosion and creating sand dunes.

Coastal dune species are adapted to tolerate the excessive heat, drought, salinity, low nutrients, and sand movement typical of coastal dune systems. The most effective plants used for building and stabilizing coastal sand dunes are long-lived perennial grasses with large, ramified root systems, flexible aboveground shoots that interfere with sand movement, and rhizomes that grow vertically and horizontally in response to sand

accretion. In the southeastern United States, sea-oats (*Uniola paniculata* L.) is the dominant sand-binding species in the exposed pioneer zone of coastal dunes (Wagner, 1964; Woodhouse, 1982). Thus, it is the primary species used for dune stabilization along the Atlantic and Gulf of Mexico coasts (Woodhouse, 1982). Plant materials for dune stabilization are obtained from other sea-oats populations. The demand for plant materials has significantly increased because of the increasing number of dune restoration projects and the large number of plants needed per unit area of stabilization. Nursery-grown sea-oats seedlings established from field collected seeds are the most reliable transplant source (Barnett and Crews, 1991; Bachman and Whitewell, 1995).

Concerns about altering the genetic constitution and population dynamics of sea-oats, and plants in general, used in restoration projects has generated a need to understand its inherent genetic variation and how that information might affect the successful development of restored dunes relative to their ecology and protection of coastal structures. The extent to which intra-specific genetic variation occurs within and between sea-oats populations has not been determined. However, variation in temperature requirements for seed production, germination, and seedling growth between Gulf and Atlantic coast sea-oats populations indicates that sea-oats populations may have diverged (Seneca, 1972a; Hester and Mendelssohn, 1991). Thus, establishing any guidelines for collecting sea-oats source materials for dune planting should be based on: (1) an understanding of the degree and patterns of genetic variation within and between populations and (2) the relevance of this diversity to plant adaptability at dune restoration sites.

A variety of molecular marker techniques are available to obtain information on population genetic composition. However, selection of molecular technique depends on specific objectives of the researcher, analytical power (resolution between individuals), knowledge requirement, time, cost, and the reliability (Hadrys et al., 1992). In population studies that use large numbers of samples, important determinants of the selection of a molecular technique are the convenience of generating extensive levels of polymorphism and the reliability of markers generated associated with their degree of reproducibility. The Random Amplified Polymorphic DNA technique (RAPD; Williams et al., 1990; Welsh and McClelland, 1990), that evolved with the advent of polymerase chain reaction (PCR) technology (Mullis et al., 1986), generates a large number of polymorphic markers more conveniently than most of the other techniques (Karp et al., 1996).

RAPD uses arbitrary primers consisting of 10-base long oligonucleotides and provides an almost endless source for thorough multi-loci screening of any organism. Advantages of RAPD are low cost, quickness, no radioisotope use, and the ability to obtain genetic information on genetically unknown organisms (Karp et al., 1996; Williams et al., 1993). The disadvantages of using RAPD for molecular studies include: (1) reproducibility problems associated with lower annealing temperatures in PCR, and (2) dominant nature of the markers rarely generating allelic information. To overcome reproducibility problems and generate reliable data, strict experimental conditions should be maintained (Karp et al., 1996; Williams et al., 1993). Thus, it would be useful if alternative techniques were developed that amplify DNA at higher annealing temperatures while maintaining the advantages of RAPD.

Molecular markers can also be used to determine the modes (sexual vs. asexual) and rates of reproduction that are responsible for the genetic makeup of a given population. Generating information on reproduction modes usually requires parental genotypic information or physical and genetic linkage maps of the species (Brown, 1989; Ritland, 1990; Kraft et al., 1998). In natural populations, parental information and linkage maps are generally not available. Genetic recombination, as revealed by molecular markers, can be used to determine the degree of genetic exchange within a population and to obtain information on asexual reproduction (Mes, 1998). The use of genetic recombination, as revealed by markers to differentiate between sexual and asexual reproduction, relies on the fact that asexual reproduction results in stable transmission of genotypes across generations without the shuffling effects of genetic recombination (Avis, 1994).

The absence of genetic recombination between individuals in clonally reproducing plants can be used to generate more accurate estimates of clones in terms of number and the degree of clonal expansion. A clone is a group of individuals, whose members (clone-mates) are genetically identical, barring mutation, and derived from a single zygote (Harper, 1977; Avis, 1994). Members of a clone differ only by acquired mutations. In clonal plants, the occurrence of somatic mutations among clone-mates has been well-documented using morphological (Hayward, 1972; Breese and Hayward, 1972), DNA sequence and molecular marker data (Caetano-Anolles, 1998 and 1999; Waycott, 1998; Silander, 1979; Primack and Kang, 1989; see review, Silander, 1985). However, the genetic variation within clones due to mutation has not been taken into consideration in many clone identification studies (genet-ramet determinations). Only the

identical individuals have been included in the analysis due to the difficulty in obtaining accurate estimates of clone-mates (Ayers and Ryan, 1997; Graham et al., 1997; Gabrielsen and Brochmann, 1998; Sydes and Peakall, 1998). As a result, the number and spatial distribution of clone-mates existing in a population are probably frequently underestimated. By detecting the lack of recombination among groups of individuals using molecular markers, we can obtain clone-mate information, which is a useful measure in clonally reproducing species to determine reproductive biology of populations.

The scope of this dissertation research included application of molecular markers to determine genetic variability in four Florida sea-oats populations located on both the Atlantic and Gulf coasts, and to consider ecological and demographic factors that may determine the genetic structures of sea-oats populations. Research was extended to develop a molecular technique to generate markers rapidly with a high degree of reproducibility and to develop an analytical approach to generate more accurate estimates of clone-mates in natural populations. These techniques may have an impact on developing new methods and avenues for population biology research. The dissertation research has been organized into three chapters:

**Molecular Analysis of Genetic Diversity in Four Florida Sea-oats
(*Uniola paniculata* L.: Poaceae) Populations: Implications for
Restoration of Dune Communities**

In this investigation, RAPD markers were used to describe the genetic structure of established adult plants and seedlings from four Florida sea-oats populations. The main research goal was to study the patterns of overall genetic variation both within and between

sea-oats populations and to make inferences on the ecological and demographic determinants of resulting population genetic structure and discuss applicability of our findings for dune restoration. Specific objectives were to determine: (1) the level of genetic variation within sea-oats populations; (2) whether sea-oats exhibit significant genetic variation between populations; and (3) whether the populations on the Atlantic and Gulf coasts exhibit distinct genetic structure.

Character Incompatibility: An Approach to Determine Reproduction Modes of Sea-Oats (*Uniola paniculata* L.) Populations

In this study, a RAPD data set generated from four adult sea-oats populations was subjected to analysis of recombination to determine the degree of genetic exchange and the occurrence of clone-mates within each population. Specific objectives were to: (1) determine the degree of recombination in sea-oats populations; (2) determine the occurrence of clone-mates and their spatial and genetic relationships; and (3) infer possible explanations for existing population differences in genetic recombination.

A Modified AFLP Technique for Rapid Genetic Characterization in Plants

The main objective of this study was to develop a molecular technique that generates markers relatively easily with high reproducibility and uniform band intensity, and higher polymorphism resulting in good analytical power. In this study, the standard amplified fragment length polymorphism (AFLP) technique was modified to develop a protocol that generated markers in fewer steps compared to the standard AFLP technique, while maintaining high reproducibility and advantages of RAPD. Using a number of

plant species, the utility of this modified AFLP technique was evaluated for the applications of molecular markers such as individual, cultivar, and ecotypic characterization. This modified AFLP technique was further compared with RAPD using four sea-oats populations to determine the efficiency in population genetic studies.

A concluding chapter, which summarizes the methodologies, results, relative importance of these findings, are outlined following the three research chapters.

CHAPTER 2 LITERATURE REVIEW

Geographical Distribution of Sea-Oats

Sea-oats, *Uniola paniculata* L., is a semi-tropical perennial grass adapted to coastal ecological conditions and thrives mainly in coastal foredunes (Wagner, 1964). Sea-oats is distributed along the Atlantic and Gulf of Mexico coasts from Virginia to northeastern Mexico, Bahamas, and Cuba (Wagner, 1964; Woodhouse, 1982). Due to physiological and morphological adaptations to extreme coastal environmental conditions such as high salinity, burial of plants by sand accretion, and high drought and heat tolerance, sea-oats dominate foredunes, especially the fore-slope and the crest of the dune (Woodhouse, 1982). In more southern sites, it outcompetes other species such as American beachgrass (*Ammophila breviligulita* Fern.) and Bitter panicum (*Panicum amarulum* Hitch. and Chase.). Sea-oats grow only in the summer period, which restricts the growing season to a few months in more northern populations (e.g. Carolinas) compared to southern regions (e.g. South Florida and Texas), where almost year-round growth has been observed (Woodhouse, 1982).

Botanical Description of Sea-Oats

The genus *Uniola* belongs to the family Poaceae. It contains nine species broadly divided into two groups. Group I contains hard-textured, xeromorphic species including

sea-oats, while group II contains soft textured woodland species (Yates, 1966). *Uniola* has two basic sets of chromosomes, 10 and 12 for group-I and group-II species, respectively. Sea-oats show 20 bivalents at diakinesis, the chromosome number being, $n = 20$ (Yates, 1966). Sea-oats show a tetraploid condition, if the basic set of chromosomes is $X = 10$ as in group I species (Yates, 1966). Sea-oats consists of creeping rhizomes, well-developed fibrous root system and aerial shoots or tillers, all of which facilitate adaptability to extreme environmental conditions. Sea-oats shoots grow from 1 to 2.5 m in height (Fernald, 1970; Westra and Loomis, 1966).

Sea-oats produce long floral culms during the spring and summer months that extend above the plant. At the upper end of the culm is the arching panicle containing many laterally comprised spikelets of 2 – 3 cm long and 8 – 10 mm wide (Colosi, 1979; Long and Lakela, 1976). Each spikelet consists of three to 20 fertile florets. However, this range can increase up to 40 based on environmental and genetic factors (Long and Lakela, 1976; Crewz, 1987). Each floret is hermaphroditic and has the potential to mature a caryopsis, ecologically equivalent to a seed (Crewz, 1987). Anthesis begins at the base of the spikelet and proceeds acropetally to the tip, while spikelet maturity occurs basipetally from tip of the branches to the base of the inflorescence (Crewz, 1987). Sea-oats seed take approximately 52 days to mature from fertilization (Colosi, 1979).

Ecological Determinants of Sea-Oats Genetic Diversity

The evolutionary forces, mutation, migration, selection, and random genetic drift are responsible for population genetic differentiation and eventual speciation in any organism (Hartl and Clark, 1997; Li, 1997). Ecological and demographic factors that regulate the rates of these evolutionary forces will determine the population genetic

structure and the degree of divergence among populations. Due to intense selection pressure, considerable adaptive genetic divergence has been observed within populations of coastal dune and marsh species. This genotypic variation is expressed in differences in growth form, reproductive potential, biomass accumulation and salt tolerance (Silander, 1979; Silander and Antonovics, 1979; Gallagher et al., 1988; Eleuterius, 1989; Pezeshki and Delaune, 1991; Seliskar and Mutz, 1993). Common environment and reciprocal transplantation studies indicate that the differences among ecotypes are adaptive and influence survival and reproduction (Silander, 1985; Gallagher et al., 1988; Eleuterius, 1989; Pezeshki and Delaune, 1991). Seliskar and Mutz (1993) have identified varieties of *Spartina patens*, *S. alterniflora* and *Distichlis spicata* that have different potentials for detritus production, decomposition rate, and below ground biomass accumulation.

Due to wide ecological distribution of sea-oats, differentiation of specific ecotypes that are suitable for different environmental conditions is expected. In fact, ecotypic differentiation with changing latitude has been reported for seed production and germination rates (Wagner, 1964; Seneca, 1972a). Main ecological and environmental factors that determine the survival of sea-oats are wind and wave action associated with storms and hurricanes, soil moisture and nutrients, salinity and salt sprays, and latitudinal effects associated with temperature and photoperiod (Wagner, 1964; Woodhouse et al., 1968; Hester and Mendelssohn, 1991).

Reproductive Mechanisms and Population Genetic Structure

Reproduction is the process that passes current genetic information to subsequent generations. Hence ecological factors and life history that affect reproduction play a major role in maintaining the population genetic structure (Lovell and Hamrick, 1984).

In plants that exhibit self-compatibility and different modes of reproduction (sexual and asexual), the resulting natural genetic variability is a function of complicated mating patterns that include a mixture of selfing, outcrossing, and asexual reproduction (Brown, 1989; Lovell and Hamrick, 1984). Owing to the environmental, demographic, and genetic factors, there exist opportunities for differences in mating systems among populations in self-compatible plants (Lloyd, 1980). Determining the influence of these ecological and genetic factors may allow estimation of breeding structure and mode of reproduction in maintaining the genetic variability in different populations.

Sea-oats reproduce by both sexual and asexual reproduction. Flowers are pollinated by wind. Wind-mediated pollination favors the possibility of outcrossing and the resulting caryopsis (grain) is also disseminated mainly by wind (Crews, 1987; Hester and Mendelssohn, 1987). Seed production may vary depending on the latitude and population size. Hester and Mendelssohn (1987) noted that more northerly and larger populations produce higher numbers of seeds compared to more southerly and smaller populations. The lower seed production in smaller populations has been attributed to lower outcrossing rates associated with fewer plants for pollination (Hester and Mendelssohn, 1987).

Vegetative propagation is prominent in sea-oats through production of tillers and horizontal rhizomes averaging a clonal spread of 0.6–1.0 m yr⁻¹ (Wagner, 1964). Thus, sea-oats populations can expand rapidly due to rapid vegetative growth. However, because sea-oats rhizomes do not fragment and transport easily, long distance dispersal mainly occurs by seed (Wagner, 1964). Therefore, clonal spread by means of rhizomes and tillers is spatially localized. However, seedlings are vulnerable to storm events due to

long establishment period (Woodhouse et al., 1968). Thus, the genetic diversity and the patterns of genetic variation may depend on the relative rates of recruitment and the fecundity of seeds and clonal spread of sea-oats genotypes as impacted by storm disturbances.

Storm Disturbance and Genetic Diversity

Disturbance is both a major source of temporal (age) and spatial heterogeneity in the structure and dynamics of natural communities and an agent of natural selection in the evolution of life histories (Sousa, 1984). Life history and temporal and spatial heterogeneity are interdependent. Temporal component derives by timely differences in patch creation causing age differences among neighboring assemblages, while the spatial component cause differentiation of these assemblages other than age such as size, microclimate etc. (Sousa, 1984). However, disturbance exerts an impact on evolutionary processes such as natural selection, population bottlenecks, local extinction, and founder effects (Sousa, 1984).

Hurricanes and storms are a major source of physical disturbance in marine and coastal ecosystems. Damage is caused by both high winds ranging from 39 to >155 mph and large waves rising from 4 to >18 feet high based on the category of hurricane and storm (Purdue University hurricane information, URL: wxp.atms.Purdue.edu/hurricane). This damage is characterized by fragmentation and removal of organisms from the substrate in marine and estuarine conditions (Coffroth and Lasker, 1998; Waycott, 1998; Kirkman, 1985). In coastal ecosystems, these damages are characterized by sand erosion, burial of organisms by sand accretion, and inundation by salt water (Woodhouse et al.,

1968; Woodhouse, 1982). Fucella and Dolan (1996) have reported that, in North Carolina, a maximum erosion loss of 70 cm sand in the middle of the sand profiles has been observed in 12-hour tidal cycle during high storm action. In the United States, significant economic and ecological damage occurs every year due to hurricanes and severe storms that originate in both the Atlantic and Pacific oceans. Between 1900 and 1996, a total of 156 Atlantic hurricanes (1.6 per year) have impacted the United States. Florida, North Carolina, Texas, Louisiana, and South Carolina are the major states impacted by hurricanes and storms (National Hurricane Center, www.nhc.noaa.gov/paststate.html).

Effect of disturbance on maintaining genetic diversity

Physical disturbance is a major mechanism that breaks the dominance of one or a few groups of organisms and generates conditions favorable for recruitment, growth, and reproduction of other organisms (Sousa, 1984). The effect of disturbance on species diversity has been explored extensively using both ecological simulation models and actual field experiments. High species diversity often present in a disturbed site is explained by two models, namely *compensatory mortality* and the *intermediate disturbance* (Connel, 1978; see review Sousa, 1984). In compensatory mortality, late successional species suffer high rates of mortality caused by disturbances, leaving resources for survival of other species. Disturbances that do not cause high mortality rates but still create resources such as space (intermediate disturbance), allow recruitment and survival of species that otherwise would be locally extinct. According to these theories, at low disturbance, competitive exclusion applies favoring the best competitive species by limiting the resources for others, while at high disturbance, some species cannot

reproduce fast enough to maintain their lost genotypes. Hence, both lead to lower species diversity. However, this intermediate disturbance hypothesis can not be universally applied in natural conditions and is case dependent (Wootton, 1998). For example, during storm disturbances, synchrony of the storm and time of seed production of different species will determine the domination by any species (Kirkman 1985).

Species composition is relatively richer in areas where the frequency of storm disturbances is high (Kirkman, 1985). Herbaceous over woody plants and clonally propagated perennial plants over annuals and biennials are reported to be more tolerant to storm events (Fahrig et al., 1993). However, few taxa have been reported to increase their number with increasing disturbance (Barbour et al., 1987).

Compared to the substantial number of studies to determine the influence of disturbance on species diversity, intra-specific studies to understand genotypic diversity are limited. Factors that may affect genotypic diversity within populations include relative levels of production, recruitment success, and survival of sexual versus asexual propagules (Hughes, 1989). This genetic diversity is a balance between generation and immigration of new genotypes, clonal growth, and local extinction from competition or stochastic processes (Sebens and Thorne, 1985).

Sebens and Thorne (1985) have developed models that can be useful in explaining the genotypic diversity that is observed at different levels of disturbances. They predict intermediate level disturbance will generate the highest levels of diversity, while increasing disturbances generate lower levels of diversity under specific conditions. Influence of disturbance on genotypic diversity depends on the species. In marine organisms with high clonal ability, such as gorgonian corals, decreased levels of diversity

have been observed with intermediate disturbances because it generates clonal propagules by fragmentation and allows better establishment success of propagules (Coffroth and Lasker, 1998). While, less clonally propagated organisms with no wide dispersal mechanisms (aggregated clones), display increased genetic diversity with increasing disturbances up to the tolerable limits of the organism (Coffroth and Lasker, 1998).

A number of plant population genetic studies have been conducted using molecular techniques for marine sea grasses including *Zostera marina* (Fain et al., 1992; Alberte et al., 1994; Ruckelshaus, 1998), *Posidonia australis* (Waycott and Sampson, 1997; Waycott, 1998), and *Thalassia testudinum* (Kirsten et al., 1998). However, these plant population studies have been focussed on genetic polymorphism and the spatial patterns of genetic diversity. None have specifically examined the impact of disturbances. Few reports have mentioned the possibility of enhanced clonal propagation and genotype mixing favored by increasing disturbance (Waycott, 1998; Kirsten et al., 1998). Promotion of outcrossing in water-pollinated *Posidonia australis* leading to high genetic diversity following high levels of wave action has also been reported (Waycott and Sampson, 1997). It is important to understand the effect of storm disturbances on maintaining population genetic structure that may have an evolutionary significance on different plant species. In particular, sea-oats populations located on the foredunes may be largely impacted by storm disturbances in areas where the storm frequency is high, subsequently influencing the genotypic diversity.

Other Determinants of Sea-Oats Genetic Diversity

Influence of population size on seed production. Sea-oats are wind pollinated, and may require large pollen pools for subsequent fertilization at the population level. Small population sizes could reduce seed production drastically owing to the lack of number of individuals contributing to the pollen pool, as reported for some of the Louisiana coast sea-oats populations (Hester and Mendelssohn, 1987). This will lead to only clonally propagated populations. Isolated clonally reproducing populations may differentiate by other forces, losing alleles through random genetic drift and accumulating new mutants (Lovell and Hamrick, 1984). Smaller populations are largely impacted by random genetic drift causing genetic differentiation among populations (Hartl and Clark, 1997). Extensive genetic differentiation could occur even between populations that are very closely located.

Seed dispersal and migration. Migration is a powerful force that prevents population differentiation (Hartl and Clark, 1997). As reviewed by Avis (1994), an average exchange of 1 individual per generation between populations is adequate to prevent extensive population genetic differentiation due to random genetic drift. Gene flow in sea-oats occurs mainly by seeds, which are generally dispersed by wind (Woodhouse et al., 1968). Wind direction and velocity, and the presence of barriers between populations limits seed dispersal. Sea-oats seeds could also be disseminated in their protective spikelets by water currents. However, there are no population genetic studies conducted to determine the direction or the degree of gene flow occurring in sea-oats.

Edaphic factors. Sea-oats seed production and seedling recruitment on different coasts and in different populations may be determined by edaphic differences such as sand texture and soil-nutrient levels (Crewz 1987). Coarser sand allows deeper light penetration favoring deeper seedling establishment that may eventually decrease seedling desiccation (Crewz, 1987). Unstable environmental conditions from recurrent storm disturbances in poor substrates would provide a selective advantage for phenotypically plastic genotypes producing more seeds that germinate sooner; seedlings having greater viability and survival; and tillers and rhizomes having rapid growth in poor substrates. Gulf coast sea-oats seeds germinate and accumulate biomass faster than do North Carolina or Atlantic seedlings, and Atlantic coast seedlings have the lowest growth potential of the three locations (Seneca 1972a). However, soil physico-chemical properties have been reported as causing little effect on the distribution of sea-oats (Hester and Mendelssohn, 1991).

Latitudinal differences and seed production. Colosi (1979) reported a lower trend of seed production in sea-oats from north to south along the Atlantic coast. Hester and Mendelssohn (1987) proposed that this trend could be the result of high humidity and summer rains associated with weak fertilization in more southern populations. Crewz (unpublished data) observed that among Florida sea-oats, average seed production per spikelet in Atlantic populations was one, and at least two in Gulf coast populations. Hester and Mendelssohn (1991) also reported a high germination rate for seeds in Louisiana populations located on the Gulf coast. Therefore, it is not clear whether ecotypic variation occurs for differential seed production based on latitude. However, Woodhouse et al.(1968) reported differential chilling requirements for seeds obtained

from the Carolinas as opposed to seeds obtained from more southerly populations. This may indicate ecotypic differentiation as an adaptation to cold conditions facilitating seed dormancy.

Applications of Molecular Markers in Population Studies

Advantages of Molecular Data

Generation of molecular marker data provides a robust method to determine genetic diversity between individuals (Karp et al., 1996). Molecular markers are preferred over anatomical or morphological data because they are phenotypically neutral, not environmentally and developmentally not regulated, and highly abundant and polymorphic (Tanksley et al., 1989). In plants, a major disadvantage of morphological markers is that the correct identity of their genotype depends on phenotypic characters, which may require a long period to achieve at plant maturity (Tanksley and Rick, 1980). Morphological markers are dominant and the traits inherited in a recessive fashion are hidden in heterozygous genotypes. In addition, epistatic interactions are inevitable in morphological markers that affect proper identification of a specific genotype because of different phenotypic expressions (Tanksley and Rick, 1980). In contrast, molecular methods use either proteins (allozymes) or DNA as the source of macromolecules and generate data that provide more accurate measures of genetic diversity than morphological data.

Allozyme data. Allozymes are the first molecular markers that were used in plant identification and population analysis (Avisé, 1994). Their use has advantages over

morphological markers such as codominant inheritance, no epistatic interaction, nonspecificity to growth stages and the presence of a large number of loci (Tanksley and Rick, 1980). Obtaining allozyme data is relatively inexpensive, direct and a large number of samples can be handled. Allozymes have been widely utilized in genetic characterization of a wide range of plant species (Giles et al., 1998; Sydes and Peakall, 1998; Waycott, 1998). However, polymorphism obtained by allozymes may be low due to lack of polymorphic loci present across particular taxa. It has been reported that more than half of the loci among average taxa lack polymorphism. Of the loci that exhibit polymorphism, the presence of more than three alleles is uncommon (Hamrick and Godt, 1989). This limitation in identification of genotypes has been reported in some plants such as seagrasses (Waycott, 1998).

DNA data. DNA based molecular assays provide a direct method to analyze the genetic makeup of any individual organism (Avis, 1994). Owing to the immense number of markers that can be generated, DNA markers facilitate detailed analyses of individual genotypes, an important aspect of population biology studies (Karp et al., 1996). In population studies, one is interested in generating data on a number of parameters such as heterozygosity, gene flow, and geographic variation. Hence, generation of data requires that more number of loci and allelic information be generated to obtain a reliable estimate of these parameters. The presence of DNA in all organisms at any growth stage and the ability to assay genotype directly are other advantages that makes DNA analysis a reliable tool. Furthermore, the requirement of DNA in minute quantities for most of the molecular techniques, its easy storage, and relatively stable nature provide less constraints when working with DNA markers. However, DNA markers may be

selectively neutral and usually cannot be used to infer fitness. However, as these markers are not subject to selection pressures, historical and geographic relationships among individuals can be drawn (Hartl and Clark, 1997).

Comparison of DNA Marker Techniques

DNA based identification of polymorphism among individuals has three major approaches. These are: 1) direct sequence analyses of nucleotide arrangements; 2) development of restriction fragments of DNA using restriction enzymes to obtain polymorphic bands; and 3) amplification of polymorphic DNA regions using polymerase chain reaction (PCR) (Mullis et al., 1986; Saiki et al., 1988). A blend of these approaches is used in recently developed methods that enables obtaining maximum genetic information with less practical constraints.

A number of molecular techniques have been developed based on the above three approaches and the genomic DNA regions that are being investigated. Major techniques are restriction fragment length polymorphisms [RFLPs], variable number of tandem repeats [VNTRs] also termed “minisatellites” (Jeffreys et al., 1985) and “microsatellites” (Tautz, 1989), random amplified polymorphic DNA [RAPDs] (Williams et al., 1990; Welsh and McClelland, 1990), sequence characterized amplified regions [SCARs] (Paran and Michelmore, 1993), amplified fragment length polymorphisms [AFLPs] (Vos et al., 1995). Labor, knowledge requirement, analytical power (resolution among samples), cost, and time inputs differ between methods. The degree of accuracy and reliability also vary with the method (Hadrys et al., 1992). Thus the choice of method depends on the availability of resources and time, purpose and the accuracy needed by the researcher.

Restriction fragment length polymorphisms (RFLPs)

RFLPs are obtained when the DNA is restriction-digested by restriction enzymes and the resulting DNA fragments differing in molecular weights among samples are electrophoretically separated according to mobility of the fragments (Avis, 1994). Once the denatured DNA is transferred to a nitrocellulose filter by southern blotting (Southern, 1975), a radiolabeled probe with known DNA sequence is then used to identify the fragment polymorphism. The probe that is used need not be identical in nucleotide sequences to the hybridizing restricted fragment. Thus the probe can be a DNA fragment that is obtained from a related taxa.

RFLP markers have been used extensively in identification and genetic characterization studies (Avis et al., 1994). RFLPs have been applied in a wide range of studies such as gene tagging, (Osborn et al., 1987), mapping (Tanksley et al., 1989) and also in phylogenetic relationships (Song et al., 1988). Advantages of this technique are, co-dominant inheritance, phenotypic neutrality, independence from growth stages, and high degree of accuracy (Yu et al., 1993). However, technical limitations of RFLPs are: (1) requirements for a good supply of DNA probes; (2) blotting and hybridization steps are time consuming and difficult to automate; and (3) relatively large quantities of DNA are required (Karp et al., 1996). Recent developments of RFLPs in association with the PCR have overcome some of these barriers. However, these methods can also be cumbersome because of the necessity for high DNA purity, sequencing, and construction of DNA libraries.

Minisatellites and microsatellites (VNTRs)

Minisatellites and microsatellites also called Variable Number of Tandem Repeats (VNTRs) are the hypervariable genomic DNA regions with tandemly arranged specific repeating units of varied lengths (Jeffreys et al., 1985 and Tautz, 1989). Size of the repeating units vary; length of repeat unit for minisatellites range from 9 to 65 bases (Jeffreys et al., 1985), while it ranges from 1 to 6 bases for microsatellites (Tautz, 1989). The pattern of repeating units is not similar between any two individuals. Thus, VNTRs are well suited to address paternity and individual testing by fingerprinting. Another advantage is that VNTRs are inherited in a mendelian fashion (Morgante and Olivieri, 1993) showing codominance similarly to RFLPs.

Of the VNTRs, minisatellites are located at many different loci having many alleles per locus. These polymorphic sites can be detected using multilocus and single locus probes. Multilocus probes consist of a few units of the repetitive sequence that detect similar sequences in many loci. They generally produce 10 - 30 bands in a southern blot (Jeffreys et al., 1985). However, due to low specificity in multilocus probes, it is difficult to generate allelic information of any specific locus. In contrast, single locus probes exhibit a high degree of specificity producing bands specific to a particular locus. To overcome the use of radioisotopes, microsatellites can be PCR-amplified and used to detect polymorphism at a particular locus. Microsatellites also called simple sequence repeats (SSR) generate ideal codominant markers for population studies because of the presence of single loci with multiple allelic information (Amos et al., 1993; Morgante and Olivieri, 1993; Soranzo et al., 1998). Microsatellite markers for population studies have been used in aquatic (Piquot et al., 1996) and forest plants

(Aldrich et al., 1998; Soranzo et al., 1998), and in animal systems (Piertney et al., 1998; Ohnishi et al., 1998). This method however, requires generating microsatellites-enriched DNA libraries and time for initial DNA sequencing and genome screening in order to develop primers flanking the microsatellite regions.

Alternatively, primers can be designed with the knowledge of SSRs to amplify the flanking DNA regions between SSRs, which are termed 'inter-simple sequence repeats' (ISSR) (Zietkiewicz et al., 1994; Gupta et al., 1994; Tsumura et al., 1996). This concept called single primer amplification reactions (SPAR) or microsatellite-primed PCR (MP-PCR) is a fairly new approach to more conveniently utilize the genetic information available in hypervariable DNA regions. Since considerable sequence knowledge on SSR sites is available, primers can be designed, screened, and selected for a specific organism to amplify the ISSR regions. The main advantage of this technique is that no prior sequencing knowledge is necessary (Gupta et al., 1994).

Random amplified polymorphic DNA (RAPD)

RAPD is obtained by PCR amplification of DNA segments using short random primers, which are, in general, 10-base long and contain arbitrary sequences (Williams et al., 1990; Welsh & McClelland, 1990). Many primers with different arbitrary sequences can be screened to generate good amplification and polymorphism that can then be used for genetic analyses. Williams et al. (1990) have shown that even single base changes on such primers generate vast differences in banding patterns compared to the previous amplification. Single primers are used in PCR with inward-oriented complementary DNA regions offering, priming sites on opposite strands. However, these priming sites must be

within the amplifiable distance by specific PCR conditions. In general, the amplification product ranges from 100 to 3000 bp (Yu et al., 1993). Polymorphism among two DNA samples is obtained due to the presence or absence of primer binding sites and/or insertions or deletions within the amplification region (Williams et al., 1990).

RAPD provides the most convenient technique to generate molecular markers. In addition to the endless source for thorough multi-loci screening, techniques that utilize arbitrary primers are less expensive, quick, and do not use radioisotopes, and can be used to obtain genetic information on genetically unknown organisms (Williams et al., 1990; 1993; Karp et al., 1996). However, owing to low annealing temperature conditions, these techniques often generate artefactual bands associated with misannealing of primers and result in poor reproducibility of the markers. Intensity of the bands between individuals can be different causing ambiguity in scoring bands (Karp et al., 1996). RAPD markers are rarely inherited as co-dominant markers.

There can also be considerable ambiguity in interpreting data obtained by RAPD. Heun and Helentjaris (1993) reported that RAPD can be subjected to “epistatic effects” i.e. one particular band can be amplified in one genetic back ground but not in another. This is explained by the varied levels of competition for primers by different templates that are present in different individuals. Finally, another complicating situation is that the bands of similar electrophoretic mobility in RAPD profiles may correspond to nonhomologous DNA sequences (Stammers et al., 1995). This restricts using RAPD to generate information among distantly related organisms. However, the RAPD technique allows production of reliable and reproducible profiles through trial and error.

RAPD markers have been used quite extensively for individual identification, genetic analysis and also in phylogenetic studies. Available literature provides ample evidence for the utility of RAPDs in population biology studies (see review; Hadrys, 1992). RAPD has been extensively used in population and conservation genetic studies of forest species (Schierenbeck et al., 1997; Schnabel et al, 1998), invasive as well as threatened plants (Sydes and Peakall, 1998; Stiller and Denton, 1995; Hollingsworth et al., 1998), aquatic plants (Kirsten et al., 1998), marsh grasses (O'Brien and Freshwater (1999; Huff et al., 1993), as well as other horticulturally and agronomically important species (Graham et al., 1996; Dowe et al., 1997; Buso et al., 1998; Ferguson et al., 1998).

Sequence characterized amplified regions (SCARs)

SCARs can be considered an extension of RAPD to detect polymorphism at a single locus and generate codominant markers (Paran and Michelmore, 1993). The amplified polymorphic bands from RAPDs are sequenced and the two ends flanking the amplified regions are used to construct new primers that consist of the random primer sequence plus some additional bases internal to the random primers. The constructed primers can then be used to detect polymorphism in the specified locus within the population. These longer primers use higher annealing temperatures, tolerate mismatching and sensitivity to PCR reaction conditions, and are known to produce amplification from both parental lines (Paran and Michelmore, 1993). However, this technique also involves sequencing and making new primers, which is time consuming and expensive when compared to RAPDs.

Amplified fragment length polymorphism (AFLP)

The most recently developed AFLP technique (Vos et. al., 1995) utilizes both principles of RFLP and techniques such as RAPDs. AFLP technique involves: 1) restriction-digestion of genomic DNA; 2) ligation of synthetic adaptors to the ends of restriction digested DNA; and 3) pre-selective and selective PCR using two primers complementary to the adaptor ligated ends generating the final products. Primers used in selective PCR are either radiolabelled or fluorescently-labelled. These final products are separated by polyacrylamide gel electrophoresis (PAGE) on a DNA sequencing gel apparatus (Vos et al., 1995).

AFLP generates an extensive level of polymorphism between individuals with high reproducibility and uniform intensity of bands. However, the AFLP technique also requires radioisotopes, and demands intense laboratory skills and is time consuming. It also generates only dominant marker information. Despite the high potential for AFLP to produce genetic information, these limitations restrict using techniques such as AFLP to only more sophisticated laboratories (Weatherhead and Montgomerie, 1991). Since the development of AFLP, it has been extensively utilized in linkage mapping and plant breeding (Maughan et al., 1996; Jones et al., 1997), population studies (Travis et al., 1996; Gaiotto et al., 1997), and also in phylogenetic research (Janssen et al., 1996).

Population Data Analysis

Sampling Strategies

Population genetic experiments often require critical thinking in how to obtain representative samples from natural populations that consist of large numbers of

individuals. Sampling primarily depends on the specific objectives and the ultimate goals of the project. These objectives may be to study population structure, identify species boundaries, or to estimate phylogenies (Baverstock and Moritz, 1996). Study of population structure reveals information on the degree of panmictic state and heterozygosity, and the patterns of geographical distribution of genetic variation. This fundamental information is of vital importance to make inferences on evolutionary forces such as mutation, migration, selection, and random genetic drift that shape these populations. However, generating information in population studies largely depends on statistics, hence sampling plays a major role in the accuracy of data. Such data constitute both multiloci and allelic information pertaining to population parameters. There are three distinct stages of sampling performed in population studies: 1) number of populations; 2) number of individuals per population; and 3) the number of loci (markers) considered. However, in terms of each sample size, a balance is required since the research projects are constrained by availability of time and money that prevents unnecessary levels of extensive sampling.

Molecular analysis tool used and population parameters tested. Sampling is also determined by the molecular technique used to generate data. Among the molecular techniques used for population studies, allozyme, microsatellite and minisatellite (VNTR), and DNA sequence data are considered codominant markers that reveal heterozygous status of the locus. In contrast, AFLP and RAPD data are dominant and produce less information. Thus, when dominant markers are used for population studies, we need larger sample sizes as compared to codominant markers. When using RAPD, Lynch and Milligan (1994) reported that two to ten times more number of individuals and

markers are required for generating similar information as RFLP. Another important factor that determines population sampling is the population parameters (e.g. average heterozygosity and gene diversity and fixation indices) tested to determine the genetic structure. Nei (1987), noted that the reliability of population parameters “P” (percent polymorphic loci) and “ n_a ” (number of alleles per locus) heavily depends on the number of individuals sampled per population. Parameters such as “H” value (average gene diversity) do not highly depend on the sample size.

Number of loci screened. In natural populations, variation in single locus heterozygosity between loci is high compared to the variation found among individuals (Nei, 1978). Therefore, the number of loci screened to generate accurate estimates of the heterozygosity is more important in electrophoretic studies. Weir (1996) suggested that at least five polymorphic loci are needed to test the significance of such data. In practical terms however, this number may require to be much higher than five. Again, the number of loci tested should depend on the power of the molecular technique.

Number of individuals. In general, intra-locus variation has been reported to adopt an L-shaped distribution caused by one allele of the locus producing a very high frequency while, other less frequent alleles in the same locus produce less variation among each other (Nei, 1987). What it means for population sampling strategy is that one could reduce the number of individuals owing to their similar intra-locus distribution. However, when the allelic differences among different population samples are smaller, it requires a greater number of individuals to detect such minor differences. Baverstock and Moritz (1996) tabulated different sampling sizes needed based on the allelic frequency (p), allele frequency difference between localities (Δp), and the statistical power of the

test. Based on their results, sampling sizes tend to increase when allelic frequency of the highest occurring allele is low and the frequency difference of alleles become smaller. Effective population size (N) and the mutation rate (u) play a major role in determining the number of individuals that should be sampled. Ewens (1972) suggested that under the infinite-site model, the number of alleles that can be detected in a sample depends on “ θ value” ($\theta = Nu$). When θ is high, we need larger sample sizes to detect alleles of a particular locus.

Number of populations. The geographical distribution of the species studied is important in understanding the number of populations to be sampled. In general, widespread species exhibit higher degrees of genetic differentiation (Hamrick and Godt, 1989) associated with variable selection pressures and restricted gene flow levels. Sampling should be done to cover the whole range of ecological distribution, however, within the limits of available funding.

Knowledge of actual population genetic structure. Actual sub-structuring of populations under study is important in determining the ultimate sample size. If the populations are under Hardy-Weinberg equilibrium, we may not need extensive sampling (Baverstock and Moritz, 1996). However, if population subdivision occurs, larger sample numbers are required to detect boundaries and gradients of subdivision. As reported by Baverstock and Moritz (1996), population sub-structuring usually remains undetected until the population with first set of sampling is tested. Thus for better estimates of population structure, we may need resampling as is generally the case for most population studies. Weir (1996) has recommended at least five localities with more than

20 individuals in each sample that includes resampling for a better estimate of population structure.

Much time and money could be saved based on our knowledge of the ecological determinants and life history factors that shape the population. For example, one major ecological determinant of the distribution of coastal plants is wind blown salt spray (Seneca, 1972a). Therefore, in coastal plant research, collection of samples ranging from high to low gradients of salt spray may reveal the gradients of genetic variability attributable to tolerance to salinity. Prior knowledge of life history factors such as mode of pollination, breeding systems, seed dispersal mechanisms, helps to improve sampling strategies and thereby the quality of the population study.

Analysis of Molecular Marker Data

Molecular techniques can be used to generate extensive amounts of polymorphic data within and between populations. There are two major types of molecular data generated: 1) qualitative or discrete data, usually generated by techniques such as RFLP, RAPD, or sequencing; and 2) quantitative data generated by DNA-DNA hybridization (Avisé, 1994). At the intra-specific level, discrete data can be used to generate population genetic information on heterozygosity and population equilibrium, mating systems, geographic variation, gene flow, and hybridization (Avisé, 1994; Karp et al., 1996). Based on the type of molecular tool used to generate data, population genetic information can be generated directly or indirectly with certain assumptions. Molecular data (both qualitative and quantitative) can also be used to generate phylogenetic relationships among individuals or taxa. A considerable number of statistical and mathematical

procedures are available to analyze molecular marker data (Weir, 1996; Hartl and Clark, 1997; Nei, 1987). Only the methods to analyze dominant marker data generated by tools such as RAPD, and the underlying assumptions needed for each method of analysis will be discussed here.

Major assumptions. RAPD and AFLP markers provide multilocus genotypic information as observed by the presence and absence of bands on electrophoretic gels. The assumptions are: homology between the bands of similar electrophoretic mobility (Stammers et al., 1995); presence of a band indicates one allele and its absence indicates the other allele of the same locus; polymorphic fragments show mendelian segregation; and each population is in Hardy-Weinberg equilibrium (Travis et al., 1996; Milligan et al., 1994).

Estimation of genetic structure and gene flow. Genetic structure of subdivided populations is readily observable in patterns of marker variation (Milligan et al., 1994). In discrete populations, the antagonistic effects of gene flow and random genetic drift regulates the distribution of genetic variation (Wright, 1978). Gene flow is usually measured by the indirect method of Wright's F-statistics (F_{ST}) (Wright, 1951), using the algorithm, $F_{ST} = 1/(4Nm+1)$. F_{ST} is used to measure genetic differentiation, which is the variance in allele frequencies among populations, σ_p^2 , standardized by the mean allelic frequency (p) at that locus: $F_{ST} = \sigma_p^2/[p(1-p)]$ (Wright, 1978; Whitlock and McCauley, 1999). F_{ST} can also be estimated using " θ ", a measure of "coancestry" (correlation of genes of different individuals), described by Weir and Cockerham (1984), which is equivalent to the F_{ST} . The value θ also provides a correction for small and unequal sample sizes and small numbers of samples (Weir and Cockerham, 1984; Travis et al.,

1996). Genetic differentiation can also be determined using G_{ST} , the proportion of total allelic diversity distributed among populations (Nei 1987). G_{ST} is calculated as $(G_{ST} = H_T - H_S)/H_T$, where H_T = the total allelic diversity at polymorphic loci and H_S = mean allelic diversity within populations.

Hierarchical analysis of population genetic variation. The above method is a more conventional way to analyze the population genetic structure. However, a hierarchical analysis of molecular variance can be completed using the squared distances between all pairs of observations (Excoffier et al., 1992; Weir, 1996). The principle behind this approach is that the conventional sum of squares can be written as the sum of squared differences between all pairs of observations (Excoffier et al., 1992). Values generated in such a manner can then be used to estimate the genetic subdivision of populations (Excoffier et al., 1992; Weir, 1996).

Determination of breeding system. Determination of breeding structure in populations is important for understanding the genetic diversity as well as the degree of inbreeding in populations (Milligan et al., 1994). Molecular markers provide a method to determine the degree of selfing and outcrossing. As reviewed by Brown (1989), the mixed-mating model is most widely used because in most plants, some degree of selfing and outcrossing occurs. The degree of outcrossing can be easily calculated when both maternal and progeny genotypic information are available (Brown, 1989). This can also be calculated with only progeny information available, however, it requires mathematical models (Ritland, 1990). Progeny analysis provides information regarding breeding systems in only the previous generation and does not necessarily express the long-term

mating system pattern (Milligan et al., 1994). In contrast, by looking at the times to common ancestry, long-term mating patterns can be elucidated (Milligan et al., 1994).

An alternative approach to obtain relative information on mode of reproduction between populations is the genetic recombination (incompatibility approach; Mes, 1998). Binary characters (morphological or molecular characters that can either be present or absent without multiple states/forms; e.g. 0/1 molecular data) can be used to obtain information on recombination by crossover events. In a population data set, the presence of two unordered binary characters with two states show recombination, if all four possible combinations (e.g. combination of character states, 11, 10, 01, and 00; simple mendelian segregation) exist between the two binary characters (Estabrook, 1972; Meacham, 1994). The use of genetic recombination, as revealed by markers to differentiate between the sexual and asexual reproduction, relies on the fact that asexual reproduction shows stable transmission of genotypes across generations without the shuffling effects of genetic recombination (Avice, 1994). The absence of genetic recombination between individuals in clonally reproducing plants, can be used to generate more accurate estimates of clones in terms of number and the degree of clonal expansion. The advantage in this method is that, it requires no allelic information and therefore, both dominant and co-dominant data generate similar information.

In summary, molecular data are useful for generating information on population genetic structure (e.g. degree and patterns of genetic variability, allelic composition and equilibrium status) and other demographic information such as reproductive patterns. Such information can be used to speculate on ecological and demographic determinants and evolutionary forces that maintain the genetic variability of populations. Even though,

sea-oats is the primary dune grass species used for dune restoration projects in the southeastern United States, the degree and patterns of population genetic variation is not known in sea-oats. However, concerns about altering the genetic constitution and population dynamics of sea-oats, and plants in general, used in restoration projects has generated a need to understand its inherent genetic variation and how that information might affect the successful development of restored dunes relative to their ecology and protection of coastal structures. DNA-based molecular assays (Avisé 1994) provide a direct method for analyzing the genetic composition of sea-oats populations. Using RAPD with an adequate number of samples, we can generate information on the degree and patterns of genetic variation, and ecological and demographic factors including the breeding structure that determine the sea-oats population genetic variability.

CHAPTER 3
MOLECULAR ANALYSIS OF GENETIC DIVERSITY IN FOUR FLORIDA
SEA-OATS (*UNIOLA PANICULATA* L.: POACEAE) POPULATIONS:
IMPLICATIONS FOR RESTORATION OF DUNE COMMUNITIES

Introduction

Coastal dune systems are often degraded by human activities that alter sand dune dynamics—e.g., by constructing buildings and cleaning beaches. Coastal beaches are also being eroded by oceanic forces resulting from rising sea level. These directional degradations of dune structure increase the likelihood of greater economic losses from hurricanes and tropical storms. Clark (1989) estimated that about 220 of Florida's 800 miles of sandy beaches were in a critical state of erosion. Continued protection and restoration of coastal dunes and high-energy beaches are necessary to minimize additional losses. Renourishing beaches with sand pumped from offshore and planting these areas with appropriate indigenous plant species are the more accepted practices for controlling coastal erosion and creating sand dunes.

Coastal dune species are adapted to tolerate the excessive heat, drought, salinity, low nutrients, and sand movement typical of coastal dune systems. The most effective plants used for building and stabilizing coastal sand dunes are long-lived perennial grasses with large, ramified root systems, flexible above-ground shoots that interfere with sand movement, and rhizomes that grow vertically and horizontally in response to sand accretion. In the southeastern United States, *Uniola paniculata* L. (sea-oats; Poaceae) is

the dominant sand-binding grass species in the pioneer zone of coastal dunes (Wagner 1964, Woodhouse 1982). Concerns about altering the genetic constitution and population dynamics of sea-oats, and plants in general, used in restoration projects has generated a need to understand its inherent genetic variation and how that information might affect the successful development of restored dunes relative to their ecology and protection of coastal structures. Although the extent of genetic variation within, and differentiation between, sea-oats populations has not previously been quantified, phenetic variations between Gulf of Mexico and Atlantic coast populations in seed weight, temperature requirements for seed production and germination, and seedling growth (Seneca 1972a, Colosi 1979, Hester and Mendelssohn 1987) suggest that sea-oats populations may have diverged genetically as well as phenotypically.

Ecological and life-history factors that affect reproductive success play a major role in determining population genetic structure (Loveless and Hamrick 1984). Plant species having mixed breeding systems (selfing and outcrossing) and mixed reproductive modes (sexual and asexual) produce complicated reproductive patterns that do not permit simple predictions of the distribution of genetic variation among and within their populations. Low genetic variation may result from asexual reproduction or from autogamy (Avisé 1994). In mixed-breeding-system species, mating patterns may vary substantially among populations as a function of environmental, demographic, and genetic factors (Lloyd 1980). Under these conditions, determining the contributions of different reproductive mechanisms in maintaining genetic composition is not possible by looking at only established adult plants in a population. Comparing the genetic structures of established adult plants and seedlings permits us to address the relative importance of reproductive modes and breeding systems and the proportion of genetic variability, represented by seedlings, contributed to the next generation through sexual reproduction.

DNA-based molecular assays (Avisé 1994) provide a direct method for analyzing the genetic composition of sea-oats populations. DNA markers are preferred over

morphological and cytological markers because DNA analysis reveals more variation (Karp et al. 1996). This variation facilitates a fine-scale analysis of individual genotypes, an important aspect of population biology studies. Compared to other techniques involving DNA sequencing and radioisotopes, the RAPD technique (Random Amplified Polymorphic DNA; Williams et al. 1990, 1993; Welsh and McClelland 1990) is a relatively quick and easy method for studying components of genetic variation (Hadrys et al. 1992, Dawson et al. 1995) in plant systems (e.g., Huff et al. 1993, Kirsten et al. 1998).

In this paper, we describe the genetic variation and differentiation, using RAPD markers, of established adult plants and seedlings from four Florida sea-oats populations, and we discuss patterns of genetic variation within and among sea-oats populations. Specifically, we wanted to address three questions: (1) what are the levels of genetic variation within adult and seedling plants of sea-oats populations; (2) are sea-oats populations genetically differentiated with regard to the adult and seedling components; and (3) do sea-oats populations from the Gulf of Mexico and Atlantic coasts of Florida have characteristic genetic structures? With respect to answers to these questions, we will consider ecological and demographic factors that may determine the genetic structures of sea-oats populations and the applicability of this information to coastal sand dune restoration.

Materials and Methods

Study Sites and Population Sampling

Adult-Plant Selection. Sea-oats shoots and rhizomes were collected from four large populations, two from the Gulf coast and two from the Atlantic coast of Florida (Fig. 3.1). The Gulf coast sites were in northwest Florida (St. George Island State Park [SG]; $29^{\circ}39'20''$ N / $084^{\circ}52'53''$ W; collection date, Oct. 1, 1996) (Fig. 3.2a), and west-central Florida (Egmont Key National Wildlife Refuge [EK]; $27^{\circ}35'15''$ N / $082^{\circ}45'45''$

W; collection date, Sept. 19, 1996) (Fig. 3.2b). The two sites from the Atlantic coast are in northeast Florida (Anastasia State Recreation Area [AN]; $29^{\circ} 53' 15''$ N / $081^{\circ} 17' 23''$ W; collection date, Oct. 3, 1996) (Fig. 3.2c) and east-central Florida (Sebastian Inlet State Recreation Area [SI]; $27^{\circ} 46' 42''$ N / $080^{\circ} 29' 27''$ W; collection dates, Oct. 15, 1996 and Mar. 20, 1997) (Fig. 3.2d). We selected the Gulf and Atlantic coast sites on the basis of their corresponding latitudes and similar population sizes. All sampling transects were established in areas where sea-oats replanting had not occurred, as well as could be determined from agency records. At each site, a 390-meter baseline was established parallel to the foredune. Twenty-six, 12-meter transects extending landward were established at 15-meter intervals perpendicular to the foredune baseline. Plant samples were collected at four-meter intervals along each perpendicular transect. By following this sampling procedure, a total of 104 samples were collected from each of the four populations. Samples were labeled by transect and position numbers. We collected more than one shoot or rhizome at each sampling point when it could be determined that the shoots belonged to the same genet.

Plant samples were placed in labeled plastic bags and transported in coolers to the University of Florida Plant Science Facility at Gainesville, Florida, where they were cleaned and planted in five-inch pots containing a 2:1 (v/v) mixture of coarse builder's sand and Vergo Klay Mix A (Schmelzer Sales Associates Inc., Tampa, FL 33610). After being maintained in a glasshouse for 18 weeks, the plants were transferred into an unheated 50% shadehouse and after an additional six weeks into 30% shade. Plants were fertilized regularly with Peter's 20:20:20 liquid fertilizer (75 mg/l N). Twenty-three out of 104 sample collected from each of the four adult-plant populations were randomly selected and analyzed for RAPD variation.

Seedling Selection. A total of 41 infructescences (ten from each population, except SI [11]) were randomly collected from the same areas in which adult plants were collected. Sea-oats "seed" (caryopsis consisting of maternal pericarp fused to the seed)



Figure 3.1. Florida map showing approximate geographic locations of four sea-oats populations used for population genetic study. SG - St. George Island State Park ($29^{\circ} 39' 20''$ N / $084^{\circ} 52' 53''$ W), EK - Egmont Key National Wildlife Refuge ($27^{\circ} 35' 15''$ N / $082^{\circ} 45' 45''$ W), AN - Anastasia State Recreation Area ($29^{\circ} 53' 15''$ N / $081^{\circ} 17' 23''$ W), SI - Sebastian Inlet State Recreation Area ($27^{\circ} 46' 42''$ N / $080^{\circ} 29' 27''$ W).

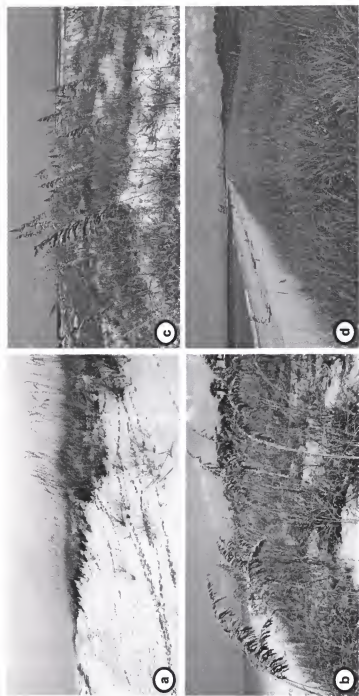


Figure 3.2. Status of Florida sea-oats population sites at the time of sampling. a) St. George Island State Park, b) Egmont Key National Wildlife Refuge, c) Anastasia State Recreation Area, d) Sebastian Inlet State Recreation Area.

were separated from the infructescences, presoaked in 100 mg/L gibberellic acid (GA_3) for 24 h in darkness, sown in plug-trays (April 22, 1998) containing Fafard soilless potting medium and then placed under intermittent mist for 6 wk after which the plug trays were transferred into a greenhouse. After 3 months, seedlings were transferred to coarse builder's sand and Vergo Klay Mix A (3:1). Each seedling was tracked relative to its population and infructescence. Eighteen seedlings were randomly selected from each population; a total of 72 seedlings from 32 of the 41 collected infructescences (7, 7, 10, and 8 infructescences from SG, EK, SI, AN) were analyzed for RAPD patterns.

Preparation of Template DNA

Adult plants and seedlings. Genomic DNA was extracted from young leaves of containerized plants using a modified method of Murray and Thompson (1980), which uses a CTAB extraction buffer. Modifications included, 1) replacing octanol with the same volume of iso-amyl alcohol, and 2) precipitation of DNA by 100% ethanol. DNA was extracted from approximately 0.1 g of fresh plant tissue in 750 μ l of CTAB buffer and was dissolved in 100 μ l of TE buffer (Sambrook et al. 1989). Seedling genomic DNA for RAPD analysis was extracted from leaf tissues of actively growing seedlings. Approximately 0.1 g of fresh tissue ground in liquid nitrogen was extracted using DNeasy plant DNA miniprep kits (Qiagen Corporation, Alameda, California).

DNA Amplification and Gel Electrophoresis

The following procedures were the same for adult plants and seedlings. Polymerase Chain Reaction (PCR; Mullis et al. 1986) conditions and thermal cycling were optimized in preliminary experiments prior to primer screening for developing amplified DNA profiles. Optimized PCR amplification solution contained PCR reaction buffer (50 mM KCl, 10 mM Tris HCl [pH 9] and 0.1% Triton X-100), 2.5 mM $MgCl_2$,

200 μ M deoxyribonucleotide phosphates (dNTPs), 2.5 pmol 10-mer random primers, 1 μ l template DNA (15 ng), and 0.3 units of Tbr DNA polymerase, all in a final volume of 15 μ l. Tbr DNA polymerase is isolated from thermostable bacterial strain, *Thermus brochianus* F500. Optimized thermal cycling was 94°C/2 min; 3 cycles of 94°C/25 sec 35°C/25 sec and 72°C/2 min; 40 cycles of 94°C/25 sec, 37°C/25 sec and 72°C/2 min; final extension time of 72°C/7 min. Eighty arbitrarily selected random primers (10-base oligonucleotides) from Operon series B, L, AJ, and AK (Operon Technologies, Alameda, CA) were screened for DNA amplifications generating reproducible results with unambiguous banding patterns. PCR reactions were repeated with the selected primers to obtain reproducible banding patterns.

Gel electrophoresis used in this study differed from standard agarose gel electrophoresis. PCR products were electrophoresed in a 3% agarose-synergelTM (1.725 g; 1.05 g dissolved in a total volume of 150 ml) in 0.5X TPE buffer (Perlman, 1991; Diversified Biotech, Boston, MA 02132; Sambrook et al., 1989). The use of a Synergel:agarose mixture allowed better separation of bands containing fragments with nearly similar molecular weights and also allowed longer run times without band diffusion and skewed patterns. Bands with 10 bp differences in the 500 bp range of fragment molecular weights could be easily separated using this electrophoresis mixture; this permitted detection of more polymorphic bands. Twenty lanes in a 15 cm² x 15 cm² gel were electrophoresed for 10 hrs at 70 volts. Each lane contained 8 μ l of PCR reaction solution mixed with 2 μ l of loading buffer III (0.25% Bromophenol blue, 15% Ficoll; Sambrook et al. 1989). Groups of four individuals from each of the four populations were run on each gel. Gels were stained in 0.024% ethidium bromide and visualized under UV light.

Band Recognition and Data Analysis

Data analysis using RAPD markers is based on certain assumptions: similar-molecular-weight DNA fragments from amplified profiles correspond to the same regions of genomic DNA; presence of a band indicates one allele, and its absence indicates the other allele of the same locus (assuming no ploidy effects; sea-oats is tetraploid [Yates 1966]); polymorphic fragments show Mendelian segregation; and each population is in Hardy-Weinberg equilibrium.

Amplified RAPD profiles were visually screened to identify monomorphic and polymorphic bands. Easily distinguishable and reproducible bands in the RAPD profiles were scored. Singleton bands (bands present in only one individual for all four populations) were not counted because they may be extremely rare. The presence and absence of bands were assigned values of 1 and 0, respectively, and binary-data matrices were developed separately for each primer and population. The software programs POPGENE, version 1.21 (Yeh et al. 1997) and TFGPA, version 1.3 (Miller 1997) were used to analyze our binary data.

The proportion of polymorphic loci (P) for each population was estimated using a 95% criterion for the most frequent allele (Nei 1987). Average heterozygosity (H) was estimated using Nei's unbiased heterozygosity estimate determined with the formula, $H = \sum h_j / r$, where h_j = the heterozygosity value for the j th locus and r = the total number of loci. Estimates were made for each individual population and for all four populations combined (i.e., species-wide). Significance differences between populations were determined using the Tukey-Kramer Multiple Comparison procedure (Hochberg and Tamhane 1987) with $\alpha = 0.05$.

Measures of genetic differentiation were calculated for each population, for population groups, and for the four populations combined. Population groups (a group is an a priori-assigned combination of populations) exhibiting the lowest within-group

genetic variation were determined by testing all possible population combinations having balanced sample sizes (e.g., population groups from each coast or latitude). The F-statistic (F_{ST}) (Wright, 1951) used to measure genetic differentiation is the variance in allele frequencies among populations, σ_p^2 , standardized by the mean allelic frequency (p) at that locus: $F_{ST} = \sigma_p^2 / [p(1-p)]$ (Wright, 1978; Whitlock and McCauley, 1999). F_{ST} was estimated using “ θ ”, a measure of “coancestry” (correlation of genes of different individuals), described by Weir and Cockerham (1984), which is equivalent to the F_{ST} . The value θ also provides a correction for small and unequal sample sizes and small numbers of samples (Weir and Cockerham, 1984; Travis et al., 1996). Other components of the F-statistic that describe total inbreeding and within-population inbreeding (F_{IT} and F_{IS}) cannot be calculated because we assume HW-equilibrium that essentially keeps F_{IT} and F_{IS} at θ and zero, respectively. We also calculated G_{ST} , the proportion of total allelic diversity distributed among populations (Nei 1987). G_{ST} is calculated as $(H_T - H_S) / H_T$, where H_T = the total allelic diversity at polymorphic loci and H_S = mean allelic diversity within populations. Gene flow among populations was calculated using the formula, $Nm = (1 - F_{ST}) / 4F_{ST}$ (Wright 1951), where N is the effective population size and m is the proportion of migrants that replace individuals in the population (Slatkin 1985).

A hierarchical analysis of genetic variation within and between populations and between groups of populations was computed using analysis of molecular variance (AMOVA). Euclidean distances (Excoffier et al. 1992) were used in computing squared-distance matrices. The F-test (Weir 1996) was used to identify parametric differences in the data ($\alpha = 0.05$). We also analyzed variance components with a Permutation test (1000 permutations; Excoffier et al. 1992), which provides ϕ values (the ϕ_{ST} estimate is considered an analog of F_{ST}) that are nonparametric estimates of significant differences. A hierarchical approach permits evaluation of sea-oats genetic variation at three levels: population groupings (i.e. species-wide), between populations, and within populations.

A principal coordinates analysis (software program NTSYS-pc, version 2.02; Rohlf 1998) was used to graphically express the between-group and within-group relationships of adult plants and seedlings from the four sea-oats populations. Nei's minimum distances (Nei 1972) were used to generate distance matrices, which were then used to calculate principal coordinates values. Using the same distance data, we generated dendrograms for adult plants and seedlings for the four populations.

Results

RAPD Banding Patterns

Adult Plants. Of 80 random primers screened, 10 were selected as producing bands with adequate resolution, reproducibility, and polymorphism to distinguish samples. Sizes of the amplified fragments selected for the analysis ranged from 0.5 to 3 kb for all the primers. After removing singleton bands from all four populations, a total of 101 bands that included 54 polymorphic bands remained for genetic analysis of adult plants (Table 3.1). Bands polymorphic in one population were often fixed in the others. RAPD profiles revealed a variety of banding patterns within populations of adult plants (Fig. 3.3a). Distinct marker bands unique to only one population (private bands) occurred only in SG and EK adult plants. SG had the most private bands (3) and the greatest diversity of RAPD patterns. Gulf- and Atlantic-coast sea-oats populations were easily distinguishable, with unique marker bands common to the two populations from each coast (Fig. 3.3a). More polymorphism and private bands were observed in RAPD profiles from the Gulf coast adult plants (14) than in those from the Atlantic coast (5); the two coasts had a total of 19 private bands differentiating them (Table 3.1).

Seedlings. The same ten primers used for adult-plant analysis were also used to produce RAPD patterns for seedlings (Fig. 3.3b). RAPD profiles for seedlings produced a total of 109 bands (Table 3.1). For the 72 seedlings, 71 different banding patterns were produced, with only two seedlings from AN having identical patterns. All four-seedling populations had a total of 58 polymorphic bands, four more than the adult plants. The adult plants and seedlings had the same proportion of polymorphic bands (53%). Banding-pattern differences among populations were greater than within populations. The greatest diversity in seedling RAPD patterns occurred in Gulf coast populations, but only SG seedlings contained private bands (2). When grouped on the basis of coast, however, the Gulf coast seedlings had over three times as many private bands as the Atlantic coast group (18 bands vs. 5 bands). The number of unique bands between coasts (23) indicates substantial genetic divergence in the seedling component. The 23 private-band differences for seedlings indicates that sexual reproduction produced greater between-coast differentiation than was represented in the adult-plant population (19 private-band differences).

Population Descriptive Statistics

Adult Plants. Within populations, the proportion of polymorphic loci (P) ranged from 20.79% to 36.63%, and average heterozygosity (H) ranged from 0.08 – 0.14 (Table 3.2). SG had the highest P and H (36.63% and 0.14, respectively) values. Gulf coast populations had significantly higher P and H values than did Atlantic coast populations. The four populations compared as a group (i.e., species-wide) had significantly more polymorphism and heterozygosity ($P = 52.47\%$; $H = 0.19$) than those of any single population.

Genetic differentiation (F_{ST}) between adult-plant populations ranged from 0.06 to 0.60 (Table 3.3). SI and AN populations on the Atlantic coast were the least differentiated from each other ($F_{ST} = 0.06$, $G_{ST} = 0.04$); EK and SG on the Gulf coast

Table 3.1. Random primers used for RAPD-PCR, and the categorical band data generated by each primer separately for four adult and seedling sea-oats populations and two Florida coasts.

Primer No. *	sequence (5' to 3')	Adult Plant Populations						Seedling Populations												
		No. Bands		Private bands		Coast	No. Bands		Private bands		Coast									
		Total	Polymorphic	Population	EK		SG	SI	AN	Total		Polymorphic	Population	EK	SG	SI	AN			
B5	TGCGCCCTTC	13	3	-	-	-	-	-	-	-	8	1	-	-	-	-	-	-	-	-
B8	GTCCACACGG	8	5	-	1	-	-	1	1	-	9	5	-	-	-	-	2	1	-	-
B2-	GGACCCCTTAC	9	4	-	-	-	-	3	-	-	9	4	-	-	-	-	3	-	-	-
L1	GGCATGACCT	12	9	-	-	-	-	3	1	-	13	10	-	-	-	-	4	-	-	-
L3	CCAGCAGCTT	12	5	-	-	-	-	2	-	-	14	6	-	-	-	-	2	1	-	-
L9	TGCGAGAGTC	8	5	-	-	-	-	2	1	-	8	5	-	-	-	-	2	-	-	-
L18	ACCACCCACC	10	4	-	1	-	-	1	1	-	11	7	-	1	-	-	2	1	-	-
AJ2	TCGCACAGTC	10	6	-	-	-	-	-	-	-	8	6	-	1	-	-	2	-	-	-
AJ4	GAATGCGACC	8	5	-	-	-	-	-	1	-	16	10	-	-	-	-	1	2	-	-
AK6	TCACGTCCCT	11	8	1	1	-	-	2	-	-	13	4	-	-	-	-	-	-	-	-
Total		101	54	1	3	-	-	14	5	-	109	58	-	2	-	-	18	5	-	-

EK – Egmont Key; SG – St. George Island, SI – Sebastian Island, AN – Anastasia; * Operon series (Operon Technologies, Inc.)

EK – Egmont Key; SG – St. George Island, SI – Sebastian Inlet, AN – Anastasia; * Operon series (Operon Technologies, Inc.)

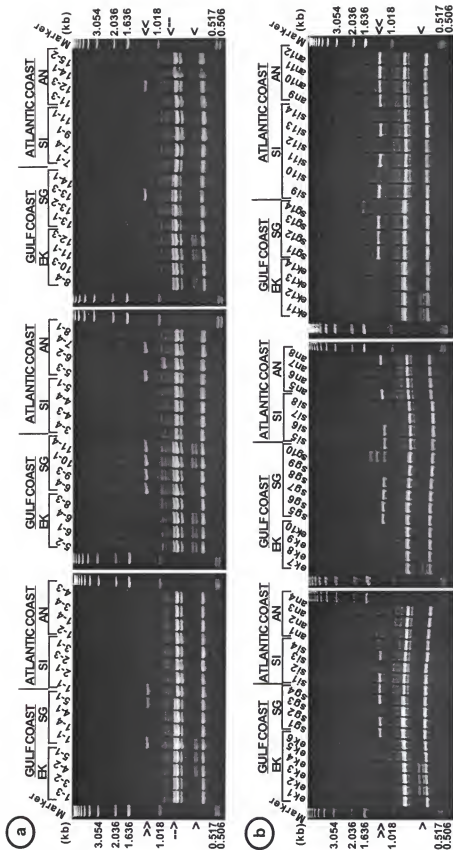


Figure 3.3. PCR amplification profiles generated using random primers OPB 20 for, (a) adult-plant and (b) seedling samples obtained from four sea-oats populations in Florida. → indicate monomorphic private bands specific to only a given coast. > indicate polymorphic private bands present only in the Gulf coast populations (EK and SG). >> indicate a polymorphic band present in SG, SI and AN populations. EK - Egmont Key, SG - St. George, SI - Sebastian Inlet, AN - Anastasia.

were more differentiated from each other ($F_{ST} = 0.21$; $G_{ST} = 0.13$) by a factor of over 3, compared to the Atlantic populations. All other pairwise population comparisons (i.e., EK with SI or AN; SG with SI or AN) produced substantially higher values ($F_{ST} = 0.58 - 0.60$; $G_{ST} = 0.42 - 0.44$). When the four populations were compared as a group (i.e., species-wide), they displayed a high level of genetic differentiation ($F_{ST} = 0.50$; $G_{ST} = 0.44$).

Gene flow, based on N_m and expressed as number of migrants per generation, was substantial between adult populations on the same coast; other population combinations experienced minimal gene flow (Table 3.3). The highest migration rate (almost four individuals each generation) occurred between SI and AN on the Atlantic coast; EK and SG on the Gulf coast had a migration rate between them of approximately one individual each generation. Migration rates for any population combinations between the two coasts were low ($N_m = 0.17 - 0.18$; approximately one individual every five to six generations).

Seedlings. For the four populations, the proportion of polymorphic loci (P) in seedlings ranged from 14.68% to 35.78%, and average heterozygosity (H) ranged from 0.05 to 0.15 (Table 3.2); the four populations combined had $P = 47.71\%$ and $H = 0.18$. SG seedlings had the highest P (35.78%) and H (0.15), almost equivalent to that of the four populations compared as a group, and differed significantly from the other populations. The Atlantic coast populations, AN and SI, had the lowest P and H values. Although EK seedlings had greater P (25.69%) and H (0.10) than the Atlantic coast populations did, the values did not differ significantly from them or from SG on the same coast.

Genetic differentiation (F_{ST}) between seedling populations ranged from 0.11 to 0.74 (Table 3.3). As with the adult plants, SI and AN seedling populations on the Atlantic coast were the least differentiated from each other ($F_{ST} = 0.11$, $G_{ST} = 0.07$). EK and SG

Table 3. 2. Average heterozygosity (H) and percent polymorphism (P) values obtained for adults and seedlings from four Florida sea-oats populations. Each individual population value was estimated using 23 and 18 samples for adult plants and seedlings, respectively. Total population values were estimated by pooling all the individual samples.

Population	Adult plant populations		Seedling populations	
	P	H	P	H
EK	29.70	0.12	25.69	0.10
SG	36.63	0.14	35.78	0.15
SI	20.79	0.08	16.51	0.07
AN	20.79	0.08	14.68	0.05
Total Populations	52.47	0.19	47.71	0.18

EK – Egmont Key, SG – St. George Island, SI – Sebastian Inlet, AN – Anastasia

Table 3.3. Measures of population genetic differentiation between four Florida sea-oats populations obtained for adult plants and seedlings, as revealed by F-statistics (F_{ST}), G-statistics (G_{ST}), and gene flow (Nm).

Population comparison	Adult plant populations			Seedling populations		
	F_{ST}	G_{ST}	Nm	F_{ST}	G_{ST}	Nm
EK - SG	0.21	0.13	0.97	0.29	0.18	0.62
EK - SI	0.60	0.44	0.17	0.70	0.54	0.11
EK - AN	0.59	0.44	0.17	0.74	0.59	0.09
SG - SI	0.58	0.43	0.18	0.55	0.43	0.20
SG - AN	0.58	0.42	0.18	0.60	0.43	0.17
SI - AN	0.06	0.04	3.76	0.11	0.07	1.94
Total Populations	0.50	0.44	0.25	0.58	0.51	0.19

EK – Egmont Key, SG – St. George Island, SI – Sebastian Inlet, AN – Anastasia

populations on the Gulf coast were more differentiated from each other ($F_{ST} = 0.29$; $G_{ST} = 0.18$) by a factor of almost three, compared to the Atlantic populations. All other pair-wise population comparisons (i.e., EK with SI or AN; SG with SI or AN) were greater ($F_{ST} = 0.55$ – 0.74 ; $G_{ST} = 0.43$ – 0.59). When the four populations were compared as a group (i.e., species-wide), they displayed a high level of genetic differentiation ($F_{ST} = 0.58$; $G_{ST} = 0.51$). These seedling values were generally higher than those for adult plants, with the exception of differentiation between SG and SI, where values were similar.

Gene flow (Nm) for seedlings ranged from 0.09 to 1.94 (Table 3.3). As with the adult plants, the greatest gene flow occurred between seedling populations on the Atlantic coast (approximately two individuals each generation). Gene flow between the Gulf coast populations (approximately one individual every two generations) was about 25% of that occurred between the Atlantic coast populations. All other pair-wise population comparisons between the two coasts (i.e., EK with SI or AN; SG with SI or AN) produced low gene-flow rates ($Nm = 0.09$ – 0.20 ; approximately one to two individuals every 10 generations). Gene-flow rates between seedling populations are consistently lower than the rates for adult plants—approximately half as many migrants.

Analysis of Molecular Variance

Adult Plants. The nested analysis of molecular variance (AMOVA) indicated that the total population variance for adult plants was almost equally distributed into the between-population (50.1%) and within-population (49.9%) components (Table 3.4). Of all possible groups tested, the least within-group variation occurred when populations were categorized based on coast. With this hierarchical analysis, the grouping for coasts explained 48.8%, within-population variation explained 41.8%, and between-population variation within the coast groupings explained 9.4% of the total genetic variance. A

higher proportion of variance indicates a greater genetic differentiation between population categories. The analysis indicates that considerable variation exists between coasts and within populations, but not between populations on the same coast.

Other groups tested produced genetic variances greater than 75% (e.g., population groups separated by latitude = 77.11%) and produced negative variances for the group component as well. These results indicate a large genetic variance between populations within groups when the populations are categorized other than on the basis of coast. In any of the hierarchical analyses that included different population groups, none were significant for the group component ($p > 0.05$), although the group based on coast was nearly significant ($p = 0.09$; Table 3.4).

Seedlings. Based on the AMOVA (Table 3.5), distribution of total genetic variance into the intra- (39.3%) and interpopulation (60.7%) components was unequal for seedlings, unlike the allocation in the adult-plant RAPDs, which was almost equal. Nested analysis using coasts as a group showed that 57.3% of the total genetic variance for seedlings was due to coastal differences, and only 10.9% resulted from differences between the populations in each coastal group. The remainder of the total genetic variance (31.8%) occurred within populations.

The lack of significance between coasts, based on the F-test, for either adult-plant or seedling component may partially result from the low number of groups (two) in the analysis, resulting in only one degree of freedom (Tables 3.4, 3.5). The nonparametric Permutation test indicated that the coastal differences were significant for seedlings but not for adult plants ($\phi_{CT} = 0.50$, $p < 0.001$ and $\phi_{CT} = 0.49$, $p = 0.148$, respectively). The lack of significance in adult plants when the ϕ_{CT} value is almost identical to that of the seedling value is likely due to the larger allocation of genetic variance to the seedling intercoast comparison (60.7%) than to the adult-plant comparison (50.1%). However, the permutation test results obtained for seedlings and adult plants cannot be compared because, these studies involved data sets generated by two different sets of samples with

Table 3.4. Hierarchical distribution of genetic variation using analysis of variance for 92 adult plants sampled from four Florida sea-oats populations. Data analysis is based on 101 RAPD markers. Data were analyzed using AMOVA version 1.55. Significance of each source of variance was tested by standard F- test and by 1000 permutations.

Source	D.f.	SSD	MSD	Variance*	F value†	Ø Statistics†
Nested analysis						
Between groups	1	319.35	319.35	6.22 (48.8%)	9.6 (0.09)	$\Phi_{CT}=0.49$ (0.148)
Populations/groups	2	66.30	33.15	1.21 (9.4%)	6.34 (0.0027)	$\Phi_{SC}=0.19$ (<0.001)
Within populations	88	468.96	5.33	5.33 (41.8%)		$\Phi_{ST}=0.58$ (<0.001)
Between Populations						
Between populations	3	385.65	128.55	5.35 (50.1%)	24.12 (<0.001)	
Within populations	88	468.96	5.33	5.33 (49.9%)		
Between Groups						
Between Groups	1	319.35	319.35	6.81 (53.4%)	53.17 (<0.001)	
Within groups	90	535.26	5.95	5.95 (46.6%)		

* percent total variances are given in parenthesis; † p values are given in parenthesis

Table 3.5. Hierarchical distribution of genetic variation using analysis of variance for 72 seedlings sampled from four Florida sea-oats populations. Data analysis is based on 109 RAPD markers. Data were analyzed using AMOVA version 1.55. Significance of each source of variance was tested by standard F- test and by 1000 permutations.

Source	d.f.	SSD	MSD	Variance*	F value [†]	Ø Statistics [†]
Nested analysis						
Between groups	1	342.86	342.86	8.58 (57.3%)	10.1 (0.086)	Ø _{CT} =0.50 (<0.001)
Popultions/group	2	68.22	34.11	1.63 (10.9%)	7.2 (<0.005)	Ø _{SC} =0.26 (<0.001)
Within population	68	323.78	4.76	4.76 (31.8%)		Ø _{ST} =0.68 (<0.001)
Between Populations						
Between population	3	411.08	137.03	7.35 (60.7%)	28.78 (<0.001)	-
Within population	68	323.78	4.76	4.76 (39.3%)		
Between Groups						
Between Groups	1	342.86	342.86	9.37 (62.6%)	61.23 (<0.001)	
Within groups	70	392	5.6	5.6 (37.4%)		

* percent total variances are given in parenthesis; † p values are given in parenthesis

different numbers (92 adult plants compared to 72 seedlings). The high degree of differentiation between coasts, based on F_{ST} and G_{ST} , the principal coordinates analysis (see below), and the dendrograms indicates the variance should be significantly different based on the coast grouping.

Principal Coordinates and Dendrogram Analysis of Genetic Distributions

Adult Plants and seedlings. Principle coordinate analysis for the adult plants and seedlings explained approximately 88% and 62% of the total genetic variance in the first three axes (eigenvectors). Both coordinate graphs showed similar patterns for the adult and seedlings (Figures 3.4a, 3.4b). The EK and SG populations from the Gulf coast diverged into two proximal groups, except for a few individuals. The two Atlantic coast populations clustered into a less diverse, single group. Genetic variation among individuals from the Gulf coast was greater compared to that of plants from the Atlantic coast. Although the seedlings produced similar genetic variability patterns to that of adults, Atlantic coast seedling populations were more narrowly distributed, with the SI and AN samples forming a tight group (Figure 3.4b). The Gulf coast seedling populations, even though proximally distributed, diverged more from each other than they did for the adult plants.

The dendrograms based on Nei's minimum distances for 92 adult plants (Fig. 3.5a) and 72 seedlings (Figure 3.5b) clearly separated the Gulf coast populations from the Atlantic coast populations; it also separated the EK and SG populations into two major groups except for two samples each in both adult plants and seedlings. The Atlantic populations (SI and AN) showed no separation; instead, individuals from each site were mixed together within the same main branches of the tree. For seedlings, we also observed clustering among individuals that belonged to the same infructescence (e.g., E15, E16, E17, and E18 in EK; S16, S17, S18 in SG) (Figure 3.5b). However, clustering

based on the infructescence was rarely observed and often mixed with seedlings from other infructescences (e.g., E10, E11, E12, and E13 in EK; S8, S9, S10, and S11 in SG).

Discussion

Our results illustrate a distinct separation of adult sea-oats populations in the two coastal regions. Based on the principle coordinate graph and the dendrogram, EK and SG populations from the Gulf coast diverged into two proximal groups, except for few individuals. The two Atlantic coast populations clustered into a less diverse single group. Genetic variation among individuals from the Gulf coast was greater compared to that of populations from the Atlantic coast. Seedling genetic variation between the two coasts and between populations exhibited overall patterns similar to that of adult plants. Seedlings from the Atlantic coast populations were more narrowly distributed, with the SI and AN samples forming a tight group. However, the Gulf coast seedling populations, even though proximally distributed, diverged more from each other than they did for the adult plants, and both populations were more widely distributed.

Genetic Differentiation Between Populations

Populations between the Gulf and Atlantic coasts exhibited substantial genetic differentiation compared to the populations on the same coast. In general, greater population differentiation, based on allozyme studies of many species (Hamrick et al. 1991), occurs in autogamous species with restricted gene flow ($G_{ST} = 0.51$), in short-lived herbaceous perennials ($G_{ST} = 0.23$), and in locally distributed or endemic species ($G_{ST} = 0.25$). On average, less population differentiation occurs in wind-pollinated, outcrossed species ($G_{ST} = 0.10$) and perennials ($G_{ST} = 0.08$). The average G_{ST} value for

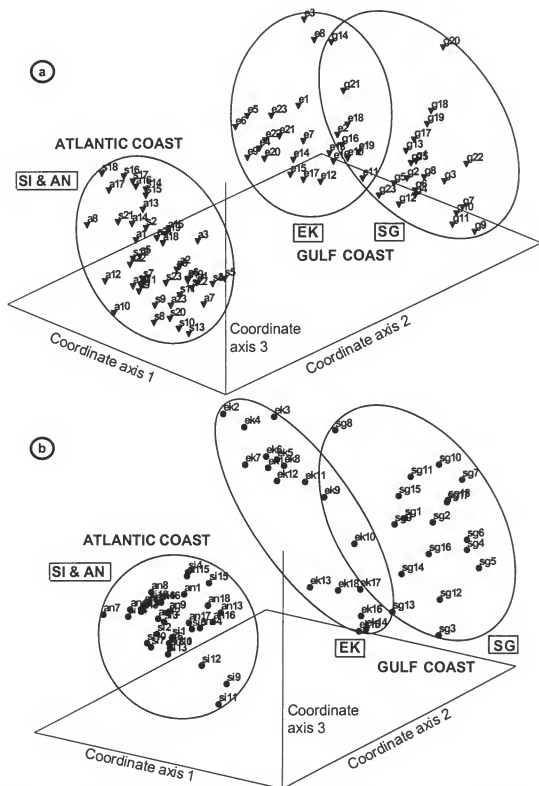


Figure 3.4. Principle coordinate graph revealing the distribution of genetic variation for (a) 92 adult-plant and (b) 72 seedling samples collected from four Florida sea-oats populations. Eigen values for the first three axes covered the 88% and 62% of the total variance distribution for adult plants and seedlings, respectively (Rohlf, 1998). EK - Egmont Key, SG - St. George, SI - Sebastian Inlet, AN - Anastasia.

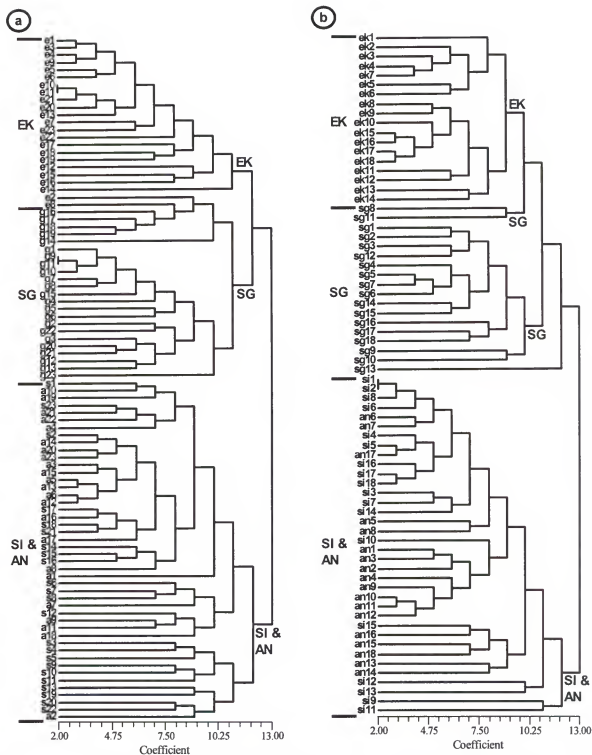


Figure 3.5. Dendrograms generated for, (a) 92 adult-plant and (b) 72 seedling samples collected from four Florida sea-oats populations. Nei's minimum distance approach (1972) was used to generate the dendrogram. EK - Egmont Key, SG - St. George, SI - Sebastian Inlet, AN - Anastasia.

our adult sea-oats populations on the same coast was 0.08, which is typical of wind-pollinated, outcrossed perennials. The average G_{ST} value for populations on different coasts was 0.43, which is similar to that for autogamous species with restricted gene flow. An exchange rate averaging one individual per generation between populations is sufficient to prevent genetic differentiation caused by genetic drift (Avice 1994). Therefore, genetic differentiation between the two coasts should result from isolation of two coastal gene pools by restricted gene flow.

The degree of genetic differentiation between populations of a species is strongly influenced by gene-flow rates. Giles et al. (1998) reported that populations of the dioecious perennial herb *Silene dioica* (L.) Clairville (Caryophyllaceae), despite its being an obligate outcrosser, are subdivided into small breeding units as a result of restricted gene flow and random genetic drift. Gene flow between sea-oats populations may occur by wind dissemination of seeds or pollen and also by rhizome fragments disseminated by water currents. However, in general, gene flow via pollen is limited to short distances (Silander and Antonovics 1979, Godt and Hamrick 1993, Avice 1994) and sea-oats rhizomes are neither fragmented easily nor transported by wind; therefore, long-distance gene flow occurs mainly through seeds (Wagner 1964, Woodhouse et al. 1968). Even though sea-oats seeds have a low tolerance of salinity (Seneca 1972a, b), seeds could be effectively disseminated in their protective spikelets by water currents.

The gene-flow characteristics of other widespread coastal species in Florida may provide insight on the distribution dynamics of sea-oats and on RAPD variation among populations. Kirsten et al. (1998) reported that populations of the tropical seagrass *Thalassia testudinum* Banks ex Kšnig (turtle-grass; Hydrocharitaceae) exhibited RAPD

variation showing genetic differentiation between a single northern Gulf population in Apalachicola Bay (sic, Apalachee Bay), Florida and three tropical populations from the Florida Keys (two) and Jamaica (one). They proposed several possible reasons for the differentiation pattern: greater reproductive success, less inbreeding, and larger population sizes in the southern populations and unidirectional gene flow from north to south.

Other Florida species that contribute to an understanding of intercoastal differentiation patterns include, *Spartina alterniflora* Loisel. (smooth cordgrass; Poaceae) and *Helianthus debilis* Nutt. (dune sunflower; Asteraceae). O'Brien and Freshwater (1999) determined from RAPD markers that smooth-cordgrass populations along the Atlantic coast of North America are clustered into three genetically different groups: New England/New Jersey (northeast), North Carolina/south Atlantic (southeast), and Gulf coast. However, a significant correlation between genetic and geographic distances was observed for Atlantic coast populations, which suggested a genetic continuum along the Atlantic coast as a result of reduced gene flow and subtle selection pressure exerted by environmental differences. The peninsular barrier between the coasts of Florida is probably more effective at reducing migration of smooth-cordgrass genes. In contrast, Campton (1992) was unable to determine intercoastal differentiation patterns based on allozymes for Florida smooth-cordgrass populations. However, a substantial degree of genetic differentiation between Florida's Atlantic and Gulf coasts, leading to the formation of subspecies, was found with allozymes in dune-sunflower populations (Wain, 1982).

Consistent, intercoastal genetic differentiation patterns in turtle-grass, smooth cordgrass, dune sunflower, and sea-oats suggest that natural-selection forces for the two Florida coasts may be consistently different. Within coasts, gene flow between populations with less physical impedance is usually sufficient to limit major genetic differentiation among populations. Sea-oats are more contiguous between SI and AN on the Atlantic coast than on the Gulf coast between EK and SG, which are separated by extensive marshes. The continuous distribution of sea-oats between our Atlantic coast populations probably accounts for the low genetic differentiation. Gene flow could also result from transfer of plants for restoration purposes, but this should be minor for the species as a whole, as it is usually discouraged by permitting agencies, although rigorous enforcement has been lacking. Distances between EK and SG (~200 mi) and between AN and SI (~150 mi) are similar. Thus, the degree of genetic differentiation does not depend solely on the physical distance between populations; it is a function of physical distance and gene-flow barriers, as well as the balance among random genetic drift, selective forces, and mutation rates.

Results of our data analyses indicated that the seedlings between populations are genetically more differentiated than adult plants. Lower genetic differentiation between populations of established adult plants compared to that of seedlings may result from vegetative reproductive predominance that leads to increased generation time, natural selection acting on seedling fitnesses and random genetic drift, all favoring population differentiation, and partial autogamy or inbreeding-depression effects on seedling fitnesses resulting in population subdivision.

Apomixis, which commonly occurs grasses (Connor 1981), can also result in population subdivision (Loveless and Hamrick 1984). However, the presence of apomixis in sea-oats could not be determined from our RAPD data, because the maternal parents of seedlings were not analyzed. Of the 72 seedlings in this study, only two individuals from one site (AN) had identical genotypes indicating the low possibility for apomixis in sea-oats. Further, our seedling data did not indicate any substructuring of seedlings due to maternal effects that would lower the genetic variation of seedlings within the same parent. Another possibility that would influence our results is, if preferential germination of outcrossed seeds to selfed seeds occurs in sea-oats. Evidence from other grasses suggests that selfed seeds may be more resistant to germination and may develop dormancy as a result of inbreeding (Ernst 1981; Billington et al. 1988).

Genetic Variation within Populations

The amount of genetic variation within different populations of a species is influenced by its breeding system, life form, propagule dispersal form and geographic range, as well as by the successional stage of each population (Loveless and Hamrick, 1984; Hamrick and Godt 1989). Allozyme variation in populations of other plant taxa has been correlated with these life history factors (Hamrick and Godt 1989, Hamrick et al. 1991). Similar correlations may be inferred from our RAPD data for sea-oats. The Atlantic coast populations (SI and AN) showed low genetic variation similar to that for populations of autogamous and endemic species ($H = 0.07$). The Gulf-coast populations, and all populations combined (i.e., species-wide) showed high genetic variation similar to the estimates for long-lived perennials, widespread species, and outcrossing to mixed-

mating wind-pollinated species (0.148 – 0.159). These estimates of population genetic diversity at the species and population levels (except SI and AN) reveal the possibility that sea-oats may be a long-lived perennial having outcrossing to mixed-breeding habit. Although we have made comparisons between allozyme- and RAPD-based genetic diversity values, levels of polymorphism estimated by these two molecular techniques usually differ for the same populations (Waycott 1995, 1998), and should be viewed with caution when interpreting the specification of breeding systems and other unknown life-history characteristics.

The amount of genetic variation within populations of sea-oats from the Gulf and Atlantic coasts may be maintained by environmental variation (micro- and macro-) specific to each population site and coast. In smooth cordgrass, for example, Daehler and Strong (1994) reported differences in seed germination rates determined by both environmental and genetic differences, subsequently exhibiting gene frequency changes in different mudflats. In species with mixed-breeding-systems, mating patterns may vary substantially among populations as a function of environmental, demographic, and genetic factors (Lloyd 1980). Silander and Antonovics (1979) observed self-compatibility in *Spartina patens* (Ait.) Muhl. (marsh-hay; Poaceae), ranging from 0% to 78% of seeds produced per culm, which demonstrates how variable breeding-system effects can be. The changes in mating patterns as influenced by micro-environmental differences may be responsible to a great deal for high and low within-population genetic variation observed for sea-oats on the Gulf and Atlantic coasts, respectively.

The difference in the frequency and severity of storm disturbances between the Florida Gulf and Atlantic coasts is a major factor that creates micro-environmental

differences between these coasts. Hurricane data (U.S. National Hurricane Center; URL:<http://www.nhc.noaa.gov/paststate.html>) reveal that 57 hurricanes struck the Florida coast between 1900 and 1996, the highest number among all states. Florida's Gulf coast experiences a greater frequency of storms and hurricanes than does its Atlantic coast. Of the four populations considered in this study, SG and EK (Gulf coast) are more frequently in the paths of hurricanes; AN and SI (Atlantic coast) have been impacted by fewer hurricanes. Dune overwash and waves during storms have created low-relief foredunes at SG (Fig. 3.2a) and a severely eroded foredune at EK (Fig. 3.2b).

The effects of disturbance on species diversity have been extensively explored with field experiments and ecological modeling (Sousa 1984, Bazzaz 1983). Kirkman (1985) reported higher seagrass diversity on the western than on the eastern Australian coast and attributed it to the greater frequency of intermediate-level storms on the western coast. High species diversity in disturbed sites may be categorized based on the frequency and severity of disturbances (Connell 1978, Sousa 1984). Compared to the many studies of disturbance on species diversity, studies on disturbance-modified genotypic diversity within populations of a species are limited. Genotypic diversity within populations is maintained as a balance among the fitnesses of life-history components such as seedling survival, balances between asexual and sexual reproduction, growth rate, within-population generation and immigration of different genotypes, and local extinction from competition or stochastic processes (Hughes, 1989; Sebens and Thorne, 1985; Bazzaz, 1983). Therefore, in our sea-oats study, the high and low genetic variability observed in Gulf and Atlantic coast populations could be the result of these

life-history factors operating at different levels, as influenced by episodic storm disturbances ranging from strong onshore winds to intense hurricanes.

Frequent storm disturbances may shift the establishment and maintenance of sea-oats populations from vegetative to sexual reproduction, consequently increasing the potential for genetic variability maintained by genetic recombination. Even though tillering and internode elongation of sea-oats is stimulated by moderate sand deposition (Woodhouse et al. 1968), erosion from storms undermines rhizomes and destroys entire populations (Hester and Mendelssohn, 1987). Under these conditions, population reestablishment may occur primarily through seedlings, depending on the establishment of a "seed bank" from the previous seasons' production or from seed production coincidental with storm disruptions. In Western Australia, for example, seagrass-seedling colonization of "blow outs" by different species was favored over vegetative expansion from indigenous seagrasses (Kirkman, 1985). The synchrony between storms and seed production periods by species determined initial seagrass-species establishment (Kirkman 1985).

Those dune species that allocate a larger proportion of their energy consumption to sexual reproduction are more likely to respond opportunistically to colonization than those that do not, (Silander and Antonovics 1979). Therefore, plants in stressful environments may be genotypically more able to colonize by seeds under stressful conditions than those from less stressful sites. The ability of sea-oats and other dune grasses to produce seeds in abundance suggests evolution for sexual-reproductive systems that accommodate high mortality rates.

The degree of seed production and dispersal, and seedling establishment specific to a site could be a factor influencing the differences in genetic variability within different sea-oats populations. Sea-oats flower during the spring and summer months. Each infructescence consists of laterally comprised spikelets (Colosi, 1979). A spikelet consists of five to 20 fertile florets, although this range could vary between three to 40 based on the environmental and genetic factors (Crewz, 1987). Each sea-oats panicle usually produces 200–400 spikelets. For Florida sea-oats, average 1996 seed production per spikelet number in Atlantic populations was 1, while, EK produced 3–4 seeds and SG produced 2–3 seeds (Crewz, unpublished data), indicating a greater seed production for populations along the Gulf coast. The efficiency of seed production (average number of seeds per spikelet divided by average number of fertile florets per spikelet) in Gulf coast populations were higher than Atlantic populations (0.21 vs. 0.14). This reproductive variation suggests a potential for greater genetic variation in Gulf coast populations.

Unstable environmental conditions from recurrent storms and disturbance exert a natural selection potential for opportunistic genotypes that produce high seed number, viability, and germination rate, and have rapid tiller and rhizome growth. In sea-oats, this selection might produce phenotypically plastic genotypes with enhanced seed production and germination rates. Sea-oats populations that were more frequently disturbed (SG and EK) produced more seeds, more efficiently. Seed germination and seedling densities are greater on the North Carolina coast in overwash areas (Hosier, 1973 cited in Hester and Mendelssohn 1987). Our preliminary seed germination trials conducted under greenhouse conditions revealed that SG and EK populations have higher germination rates (unpublished data). Hester and Mendelssohn, (1987) reported higher seed germination

rates for Gulf coast Louisiana populations, where frequency of storms is high. Even though population biology studies have not been conducted on sea-oats to support this opportunistic genotype selection hypothesis, the most preferable sites for commercial seed collection are the Florida Panhandle and the North Carolina coast (Otto Bundy, personal communication), where hurricane frequency is the highest for all Gulf and Atlantic coast populations. However, the disturbance dynamics favoring genotypes producing more propagules may be of fortuitous nature, and may limit the ability of natural selection to produce the site-specific best-adapted genotypes with other traits combined, as shown by Silander (1985) for marsh-hay.

Sea-oats seed production and seedling recruitment on different coasts and in different populations may also be determined by edaphic differences such as sand texture and soil-nutrient levels (Crews 1987), especially micronutrients and sea wrack deposited during storm overwash (Hester and Mendelsohn 1991). Unstable environmental conditions from recurrent storm disturbances in poor substrates would provide a selective advantage for phenotypically plastic genotypes producing more seeds that germinate sooner; seedlings having greater viability and survival; and tillers and rhizomes having rapid growth in poor substrates. Gulf coast sea-oats seeds germinate and accumulate biomass faster than do North Carolina or Atlantic seedlings, and Atlantic coast seedlings have the lowest growth potential of the three locations (Seneca 1972a). These differences are positively correlated with the level of substrate disturbance, based on storm frequency that prevails at each site.

The stability from disturbance in Atlantic coast populations, in contrast to those on the Gulf coast, may have caused SI and AN to reach a state of equilibrium, resulting in

fewer but more dominating genotypes. With the restricted introduction of new genotypes, autogamy will occur as a result of widely distributed ramets of the same large genet (Avis 1994), further lowering the genetic variability. Under this condition, inbreeding depression may limit zygote formation, seed germination, and seedling survival of unfit genotypes, leaving only a few genotypes producing the majority of viable offspring (Daehler and Strong 1994). In Atlantic coast populations, we found tighter clustering of seedlings compared to the adult plants that may indicate an ongoing process of lowering genotypic diversity in these sites. A stable site may also provide better habitat for granivores (e.g., mice), that consume most of the seedlings that fall to the ground. Reader (1991) demonstrated how ground cover reduces seedling production and survival because of better habitats for seed eaters.

Implications for Dune Restoration

In 1998, the Florida Legislature allocated \$30 million to repair beaches and dunes in northwest Florida damaged by Hurricane Opal and previous storms. Accelerated dune-restoration efforts in the Florida panhandle and elsewhere have significantly increased the demand for sea-oats in recent years. Ironically, the Gulf coast populations, considered to be the more consistent seedling producers, are the very ones that have been most damaged by storms. Concern for over harvesting of sensitive, storm-damaged natural stands has forced states such as Florida to impose harvesting restrictions for sea-oats plants and seedlings from the wild. These restrictions significantly limit natural sources of sea-oats and the ability of native-plant nurseries to meet demands for planting units and may encourage the use of unfit plants from remote sources. Even in areas where harvesting is permitted, establishing guidelines for collecting sea-oats for dune planting

should be based on an understanding of the degree and patterns of genetic variation within and between populations and on the relevance of this diversity to plant adaptability within dune restoration sites.

Maintaining plant genetic diversity and locally adapted genomes in restored communities are critical concerns facing restoration ecologists. The negative aspects of these concerns include, but are not limited to, altering genetic diversity when using nursery-propagated plants and using "unsuitable" genotypes collected from divergent populations distant from the planting site. The relationship between phenotypic variation and geographical distance between locations is unclear, however, and varies among species and habitats. The concern over potential limited genetic variation of remote donor populations has been addressed by permitting agencies, by restricting the distance (e.g., a 50-mile radius) plants can be moved between donor and planting sites, with the idea that local populations are more likely to have similar genetic structures and therefore similar habitat functions. This distance restriction is based on little supportive data, however, and is arbitrary, as substantial genetic variation occurs over short distances within sub-populations or between proximal populations of dune and marsh plants (Silander 1979, 1984, Campton 1992, Stiller and Denton 1995).

The Gulf and Atlantic coast sites are approximately the same distance apart, yet, compared to the Gulf coast, the Atlantic coast populations are barely differentiated, based on RAPD analysis. Therefore, the degree of sea-oats population differentiation, and for many coastal species as well, may be related more to the spatial fragmentation of populations than to actual distances. Gene flow should be lower in fragmented distributions in contrast to continuous ones. Fragmentation may be partially overcome by

enhanced gene flow in coastal species whose diaspores are dispersed via water currents, such as with mangroves, smooth cordgrass, and possibly sea-oats spikelets.

Environmental managers may interpret the genetic differences we revealed using RAPDs to suggest that translocating sea-oats plants between populations, especially between the Gulf and Atlantic coasts, may not be acceptable. Although this argument is partially valid, the relationships of genetic variation to ecological adaptation and fitness in sea-oats have not been established and may not be a concern. Genotypic variation may be expressed as phenotypic differences in growth form, reproductive potential, biomass accumulation, and salt tolerance, among others (Gallagher et al. 1988, Eleuterius 1989, Pezeshki and Delaune 1991, Seliskar and Mutz 1993, Hester et al. 1996). At this time, all we are absolutely sure about regarding our sea-oats populations is that they differ in their RAPD genetic constitutions, some more than others.

Considerations of inter-site distance and genetic differentiation among populations may be less important than poor field-collection procedures and nursery culture practices that limit genetic variation, possibly select for susceptible genotypes, and encourage epigenetic development not conducive to survival in stressful restoration sites. In particular, selection of genotypes that lack appropriate sexual and asexual reproductive characters can delay the establishment, persistence, and long-term maturation of restored communities. A counter argument for increasing the genetic diversity within a population by bringing in genetically different stock also contains valid points.

In Florida, sea-oats is the preferred dune-restoration species because it grows well in most stressful foredune environments and produces enough seedlings to be harvested

economically. Nursery-grown sea-oats seedlings, produced from field-collected seeds, are the more frequently used planting units (Barnett and Crews 1991, Bachman and Whitwell 1995). Our RAPD values for seedlings show that a randomized-collecting approach for seeds should provide ample genetic variation for local restoration sites. Large-scale sea-oats production using micropropagation (Hovanesian and Torres 1986) has not been reported, but if implemented properly, micropropagation of sea-oats could be used to select for special-purpose genotypes (e.g., highly competitive) and for ample genetic variation without damaging sensitive donor populations. Our investigation raises the possibility that selecting genetic variability appropriate to the desired restoration outcome for each site is the better approach rather than having a "one size fits all" policy based on inter-site distances.

Clearly, we have but a rudimentary knowledge of genetic variation in coastal plants and how using that knowledge affects sand dune restoration efforts. Much of the genetic variation within genomes may not result in phenotypic expression but may only be non-expressed historical genetic variation (Lowenstein 1999). Consequently, following up this initial genetic study on sea-oats with evaluations of phenotypic variation and its relation to genetic variation and the role it plays in altering dune physiognomy are the next critical steps. Even though the RAPD technique may be detecting some irrelevant variation, it is still a useful technique for investigating inter-site variation and its relation to disturbance and other environmental variation. Until the relationship between sea-oats genotypes and phenotypic expressions are evaluated, the validity of the opposing viewpoints regarding translocation of plants for restoration will remain unsubstantiated. We are continuing our research into these aspects of sea-oats

ecological fitness via evaluation of tissue-cultured strains (selected genotypes) planted in common-garden and reciprocal-transplant studies. These results will be discussed in future publications.

CHAPTER 4
CHARACTER INCOMPATIBILITY: AN APPROACH TO
DETERMINE REPRODUCTION MODE OF SEA-OATS
(*UNIOLA PANICULATA* L.) POPULATIONS

Introduction

Genetic variation is caused by genetic recombination between individuals and acquisition of mutations. Mating systems determine how much genetic variation is transmitted between generations and form the genetic makeup for future selection (Brown, 1989). Ability to estimate the mating systems active in plant populations is needed to understand the present genetic structure and to predict their future evolution. A number of different methods and models are available to detect the mode (sexual versus asexual) and rate of reproduction (see reviews: Brown, 1989; Ritland, 1990). However, these methods often require extensive allelic information in parental genotypes to detect mating patterns (Brown, 1989). Genetic recombination can be used to determine the reproduction mode since sexual reproduction is characterized by the presence of recombination whereas asexual reproduction is characterized by a lack of recombination (Avisé, 1994; Graser et al., 1996; Mes, 1998). If physical and genetic linkage maps are available, the position of cross-over events associated with meiosis can be determined to indicate recombination (Nachman, 1997; Kraft et al., 1998). However, such maps are generally not available for natural populations.

Information on genetic recombination without linkage data may be generated using the segregation patterns of unordered binary characters (e.g. morphological or molecular markers that are either absent or present) in a population data set (character incompatibility; Mes, 1998). Analysis is based on simple mendelian segregation of the characters (markers) between individuals, to obtain information on recombination by crossover events (Fig. 4.1). Two unordered binary characters show recombination if all four possible combinations of character states (11, 10, 01, and 00) exist (incompatible characters; Estabrook, 1972; Meacham, 1994; Mes, 1998). The presence of all four possibilities in a set of population data indicates that individuals exhibit genetic exchange as a result of one or more generations of sexual reproduction (Mes, 1998). This conclusion is strengthened if many marker combinations exhibit all four possibilities. Two of these combinations represent parental types, while the remaining two are recombinant types generated by genetic exchange (Fig. 4.1). In the absence of linkage between any two characters, four possible combinations of character states would be expected as a result of sexual reproduction.

It is possible that the same four combinations of two characters could be introduced into a population by parallel mutations (independent acquisition of the same character state in two or more individuals) or by introduction of individuals from outside of a population's natural range. However, any effect of parallel mutation and migration, and linkage of markers on analysis can be greatly reduced by screening a large number of loci and individuals (Mes, 1998). A lack of recombination due to asexual reproduction can also be indicated by this approach, if a large number of loci are studied (Hulst et al., 1999).

Step 1: Individuals showing presence and absence of markers

	1	2	3	4	5
marker (a)	■		■	■	■
marker (b)	■	■		■	■

Step 2: Individuals resulted from crossing between 2 and 3 in step 1

	i	ii	iii	iv
marker (a)		■		■
marker (b)	■			■

Figure 4.1. A model illustrating the use of molecular markers to generate information on genetic recombination by sexual reproduction in populations. Step 1 shows individuals 2 and 3 with missing markers a and b due to mutation, respectively. Step 2 shows all possible marker combinations of progeny individuals that could result from crossing between individuals 2 and 3 in step 1 by one or more generations of sexual reproduction. i and ii represent parental types (parent individuals 2 and 3, respectively), while iii and iv indicate recombinant types.

In plants with extensive clonal (asexual) reproductive abilities, accurate estimates of genets or clones (a group of individuals, which descended from a single sexually-produced zygote that is genetically identical barring mutation [Harper, 1977]) and ramets or clone-mates (individuals within a genet or a clone) are important to generate information on mating systems and sex ratios within mixed sexual-asexual populations (Avisé, 1994). However, accurate identification and mapping the distribution of clones in natural populations are usually difficult (Sebens and Thorne, 1985). Some methods to detect clones in natural populations are excavation of stolons or rhizomes and analysis of morphological and karyotype differences (Silander, 1985b; Sebens and Thorne, 1985). These techniques are cumbersome and labor intensive. Allozyme analysis can also be applied to detect clones, however, the utility of allozymes is limited by the lack of variable loci in many populations and developmental and environmental variation in allozyme expression (Silander, 1985b).

Sea-oats (*Uniola paniculata* L.) is a semi-tropical perennial grass adapted to coastal beach conditions and thrives mainly in coastal foredunes (Wagner, 1964). Sea-oats is distributed along the Atlantic and Gulf of Mexico coasts from Virginia to northeastern Mexico, Bahamas, and Cuba (Wagner, 1964; Woodhouse, 1982). In the southeastern United States, sea-oats is the predominant species used for dune stabilization to limit coastal erosion and hence is of considerable economic importance (Woodhouse et al., 1968; Woodhouse, 1982; Dhal and Woodard, 1977). Population genetic studies using the random amplified polymorphic DNA (RAPD) technique have been conducted to determine the genetic diversity of sea-oats that may be useful for selecting plant material and for

maintaining the genetic structure of revegetated planting sites (Ranamukhaarachchi et al., 1999).

Discrimination of genets and ramets in species that have extensive vegetative reproduction is an important step in population biology studies (Ayers and Ryan, 1997). Sea-oats reproduce sexually by seed and vegetatively by tillers and rhizomes (Wagner, 1964; Woodhouse, 1982). Pollination and seed dispersal are mediated by wind favoring the possibility of cross-pollination (Crewz, 1987; Hester and Mendelssohn, 1987). Crewz (1987) also reported the possibility of self-compatibility, although this has not been verified. Vegetative propagation is prominent in sea-oats and a rhizome spread of 0.6–1.0 m yr⁻¹ has been reported (Woodhouse et al., 1977). However, information on the occurrence and extent of genets and ramets in sea-oats and their influence on sea-oats population biology is very limited.

It is possible that sea-oats populations could be analyzed using recombination (character incompatibility approach), as revealed by DNA markers, to determine the degree of genetic exchange between individuals and identify clone-mates. Among the available DNA markers, RAPD generates a large number of polymorphic and multi-loci data that facilitates the use of recombination (character incompatibility) to analyze reproduction modes. In this study, we analyzed RAPD data generated from four natural sea-oats populations from the Florida Gulf and Atlantic coasts using character incompatibility to obtain information on the genetic diversity and clone-mates within each population. The specific objectives of this study were to: 1) determine the degree of genetic recombination in sea-oats populations; 2) determine the occurrence of clone-

mates and their spatial and genetic relationships; and 3) infer possible explanations for existing population differences in recombination and asexual relationships.

Materials and Methods

Plant Materials and DNA Extraction

Four sea-oats natural populations, two from Gulf of Mexico and two from Atlantic coast in Florida were sampled in this study. Gulf coast sites are in northwest Florida (St. George Island State Park [SG]; $29^{\circ}39'20''$ N / $084^{\circ}52'53''$ W) and west-central Florida (Egmont Key National Wildlife Refuge [EK]; $27^{\circ}35'15''$ N / $082^{\circ}45'45''$ W) (Fig. 2). The two sites from Atlantic coast are in northeast Florida (Anastasia State Recreation Area [AN]; $29^{\circ}53'15''$ N / $081^{\circ}17'23''$ W) and east-central Florida (Sebastian Inlet State Recreation Area [SI]; $27^{\circ}46'42''$ N / $080^{\circ}29'27''$ W) (Fig. 4.2). Initial population sampling was done systematically by establishing transects in areas where sea-oats replanting had not occurred. At each site, a 390-meter baseline was established parallel to the foredune. Twenty-six, 12-meter transects extending landward were established at 15-meter intervals perpendicular to the foredune transect line. Plant samples were collected at 4-meter intervals along each perpendicular transect. Thus, 104 individuals were collected from each population. Of the 104 individual samples collected, 23 were randomly selected for RAPD analysis.

From 23 randomly selected plants, young leaf tissue was used for extraction of genomic DNA. DNA was extracted using a modified method of Murray and Thompson (1980), which uses CTAB extraction buffer. Modifications included, 1) replacing octanol



Figure 4.2. Florida map showing approximate geographic locations of four sea oats populations used for population genetic study. SG - St. George Island State Park ($29^{\circ}39'20''\text{N} / 084^{\circ}52'53''\text{W}$), EK - Egmont Key National Wildlife Refuge ($27^{\circ}35'15''\text{N} / 082^{\circ}45'45''\text{W}$), AN - Anastasia State Recreation Area ($29^{\circ}53'15''\text{N} / 081^{\circ}17'23''\text{W}$), SI - Sebastian Inlet State Recreation Area ($27^{\circ}46'42''\text{N} / 080^{\circ}29'27''\text{W}$).

with iso-amyl alcohol, and 2) precipitation of DNA by 100% ethanol. DNA was extracted from approximately 0.1 g of fresh plant tissue in a 750 μ l volume of CTAB buffer. Extracted DNA was dissolved in 100 μ l of TE buffer (Sambrook et al., 1989).

PCR Amplification

Optimized PCR amplification solution contained PCR reaction buffer (50 mM KCl, 10 mM Tris HCl [pH 9] and 0.1% Triton X-100), 2.5 mM $MgCl_2$, 200 μ M deoxyribonucleotide phosphates (dNTPs), 2.5 pmol 10-mer random primers, 1 μ l template DNA (15 ng), and 0.3 units of Tbr DNA polymerase all in a final volume of 15 μ l. Tbr DNA polymerase is isolated from thermostable bacterial strain *Thermus brochianus* F500. The optimized thermal cycling was 94°C/2 min; 3 cycles of 94°C/25 sec 35°C/25 sec and 72°C/2 min; 40 cycles of 94°C/25 sec, 37°C/25 sec and 72°C/2 min; final extension time of 72°C/7 min. Eighty arbitrarily selected random primers (10-base oligonucleotides) from Operon series B, L, AJ, and AK (Operon Technologies, Alameda, CA), were screened for DNA amplifications generating reproducible results with unambiguous banding patterns. PCR reactions were repeated with the selected primers to obtain reproducible banding patterns. PCR products were electrophoresed in a 3% agarose-synergelTM in 0.5X TPE buffer (Perlman, 1991; Diversified Biotech, Boston, MA 02132; Sambrook et al., 1989). Gels were then stained with ethidium bromide and visualized under UV-light.

Data Analysis

The RAPD analysis was based on the assumptions that: 1) RAPD profiles reflect genetic variation in the entire genome, 2) RAPD patterns provide information on many unlinked loci, 3) identical genotypes are separated only by asexual generations, if many variable loci have been screened. Independent sexual events generating identical genotypes are highly unlikely (Mes, 1998). Of the RAPD bands generated in this study, monomorphic bands and polymorphic bands with only a single occurrence of presence or absence of the band were removed from the incompatibility analysis, as they do not contribute to the recombination between individuals (uninformative characters). The remaining RAPD bands in each population were then scored (1 and 0 for presence and absence, respectively) and analyzed using the software program PICA95 (Wilkinson, 1995).

To generate information on sexual and asexual reproduction, individual and total recombination counts (incompatibility counts; Wilkinson, 1995) were computed. If any two RAPD markers (with presence and absence of bands) produced all four possible combinations, this was considered as one recombination count (Wilkinson, 1995; Mes, 1998). The total recombination count for each population was computed by taking all pair-wise character comparisons and summing them to generate the total matrix. Each individual in the set shares a certain fraction of the total recombination, which was calculated as the difference in total recombination counts when a specific individual was included or excluded from the data set (Wilkinson, 1995). Whether the distribution of the character states of one character relative to another character is a result of a chance event, was tested by permutation to generate LeQuesne probabilities (LeQuesne, 1969).

Determination of Recombination and Clone-Mates

To compare the degree of genetic exchange between members within each population, individual and total recombination counts were obtained for all 23 samples. Recombination values were then plotted to obtain information on contribution of each individual towards total recombination counts. Detection of clone-mates within a population was obtained by successively removing individuals beginning with the highest recombination count (one by one in a descending order), until the remaining individuals showed zero or very low recombination counts with no further decline (Mes, 1998). This procedure was followed for each of the four sea-oats populations. Individuals with zero or very low recombination counts that remained in the data set after successive deletions may represent clone-mates with no sexual recombination. If marker differences are present between these individuals, these may be due to somatic mutations (see discussion). To determine the identical genotypes based on the RAPD data, entire RAPD patterns (consisting of 101 bands) were checked within each group of clone-mates. Total recombination counts obtained after successive deletions were then plotted to graphically represent the total recombination decrease. To determine the spatial distribution of clone-mates in each population, position of clone-mates was detected using the site-plan of population sampling. To determine genetic relationships between clone-mates, dendrograms were generated for each group of clone-mates using Nei's minimum distance algorithm (Nei, 1972).

Results and Discussion

A total number of 101 RAPD bands were generated using 10 random primers selected after screening a total of 80 primers. After removing monomorphic and uninformative polymorphic bands, the remaining bands in each population were used in the analysis. The number of informative RAPD bands for each population was: EK: 28; SG: 39; SI: 23; and AN: 19.

Determination of Recombination at the Population Level

Total recombination counts generated for EK, SG, SI and AN were 174, 498, 130 and 101, respectively. Thus, results indicated that the Gulf coast, especially SG, yielded higher total recombination counts than the Atlantic coast populations. The SG population also contained more individuals with higher individual recombination counts (e.g. S1 > 65; S2 and S13 > 35) compared to the other populations (Fig. 4.3a vs. Fig. 4.3b, 4.3c, and 4.3d). For example, in SG, 10 out of 23 individuals showed recombination counts of more than 10 (Fig. 4.3a). In contrast, only two produced individual recombination counts of more than 10 in other populations (e.g. A3 and A7 in AN [Fig. 4.3c]). The total recombination count is a result of: 1) one or a few individuals exhibiting a higher level of genetic exchange, which may result from crossing between divergent parents; or 2) all individuals subjected to analysis exhibiting moderate levels of genetic exchange (Mes, 1998). In RAPD data, divergence between individuals is indicated by the presence of polymorphism. In this study, both factors seem to be involved in SG with a few individuals generating high recombination counts and a majority of individuals exhibiting moderate levels of recombination counts compared to other populations.

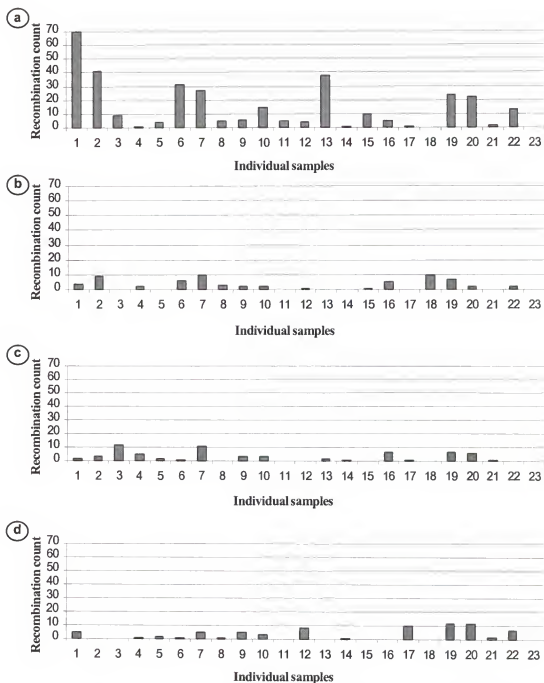


Figure 4.3. Individual recombination counts for 23 sea-oats plants from each of the four sea-oats populations. (a) St. George Island, (b) Egmont Key, (c) Anastasia, and (d) Sebastian Inlet. Absence of bars for some individuals indicates a zero recombination count.

The presence of high recombination counts and genetic diversity in SG followed by EK compared to SI and AN, may be explained by differences in the frequencies of storm events. EK and SG populations on the Gulf coast experience more storm damage compared to SI and AN populations on the Atlantic coast (United States National Hurricane data; URL: <http://www.nhc.noaa.gov/paststate.html>). The influence of ecological disturbances such as fires and storms on increased genetic diversity has been well documented in other organisms (Connell, 1978; Kirkman, 1985; see review Sousa, 1984). In sea-oats, a higher genetic variability was found for both adult plants and seedlings in the Gulf coast populations, especially SG, which has been attributed possibly to higher storm frequencies (Ranamukhaarachchi et al., 1999). Populations that are mainly established by seeds and have high genetic diversity must generate higher recombination values, because of the opportunities for genetic exchange between more diverse individuals.

High-energy ecosystems have been reported to cause population destabilization and the creation of more seed-based populations (Kirkman, 1985; Sousa, 1984). Although vegetative reproduction is prominent in sea-oats (Woodhouse, 1982), erosion during severe storms damages rhizomes and entire populations restricting the expansion by vegetative reproduction (Hester and Mendelssohn, 1987). Under these conditions, recolonization may occur primarily through seedlings. Thus, frequent storm disruptions may restrict the vegetative spread of sea-oats while enhancing more seed-based populations in EK and SG.

Detection and Spatial Distribution of Clone-Mates

Successive deletion of 23 individuals in each population generated groups of six to eight individuals with the lowest possible recombination values based on the population. These individuals at EK were, EK: E15, E17, E18, E20, E21, and E23 (total recombination count: 3) (Fig. 4.4a); SG: S9, S10, S11, S12, S14, and S23 (total recombination count: 7) (Fig. 4.5a); SI: I1, I2, I3, I4, I5, I6, I7, and I8 (total recombination count: 8) (Fig. 4.6a); AN: A18, A19, A20, A21, A22, and A23 (total recombination count: 1) (Fig. 4.7a). The low recombination counts within these groups could be caused by the absence of sexual reproduction or by the lack of variable bands to indicate recombination. Therefore, it is important to determine whether groups of individuals show low recombination because they did not: a) undergo sexual reproduction, or b) differ much in their fingerprint patterns.

Our study based on 101 markers with 54 polymorphic loci (53.5% polymorphism) showed high genetic variability between fingerprint patterns. Ellstrand and Roose (1987) reported a significant correlation between the number of characters scored and the number of genotypes found. However, we used many RAPD bands well beyond the maximum number of characters analyzed (26 loci) in their survey, and detected only relatively few identical genotypes. Thus, very low recombination counts indicated the absence of sexual reproduction between individuals in each group. These groups of individuals probably descended from a single sexually produced zygote or a genet, which consist of clone-mates.

Comparison of RAPD patterns within these groups of clone-mates indicated only two identical individuals in SG (S9 and S10) and SI (I6 and I7) populations and showed

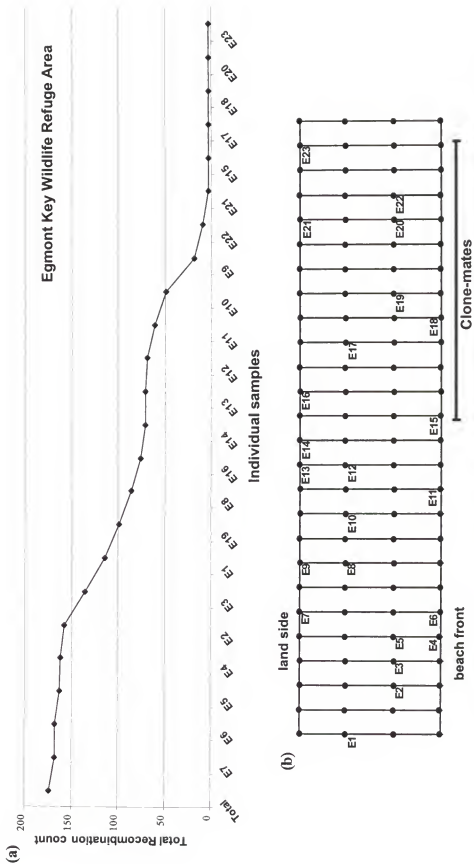


Figure 4.4. Successive deletion of sea-oats samples beginning with individuals having the highest recombination counts. a) Graphical representation of the reduction in total recombination counts against successive deletion of individuals from Egmont Key and (b) the site plan showing spatial distribution of samples (E1 - E23) and clone-mates .

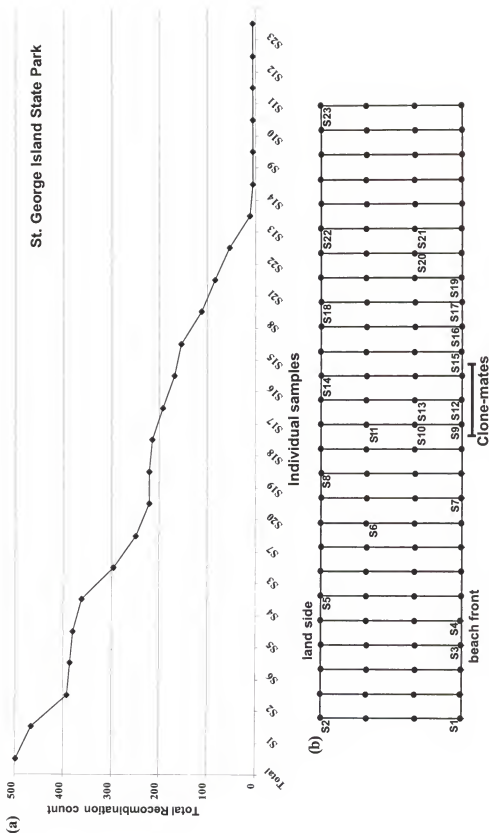


Figure 4.5. Successive deletion of sea-oats samples beginning with individuals having the highest individual recombination counts. a) Graphical representation of the reduction in total recombination counts against successive deletion of individuals from St. George and (b) the site plan showing spatial distribution of samples (S1 - S23) and clone-mates.

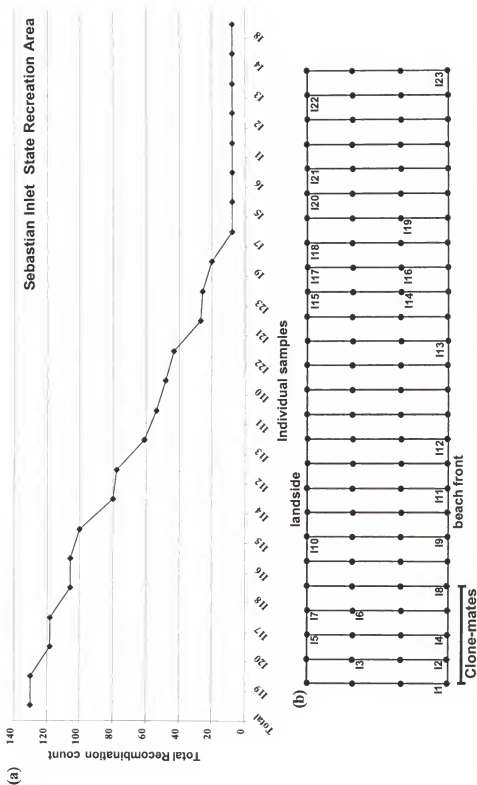


Figure 4.6. Successive deletion of sea-oats samples beginning with individuals having the highest individual recombination counts. a) Graphical representation of the reduction in total recombination counts against successive deletion of individuals from Sebastian Inlet and (b) the site plan showing spatial distribution of samples (11 - 123) and clone-mates.

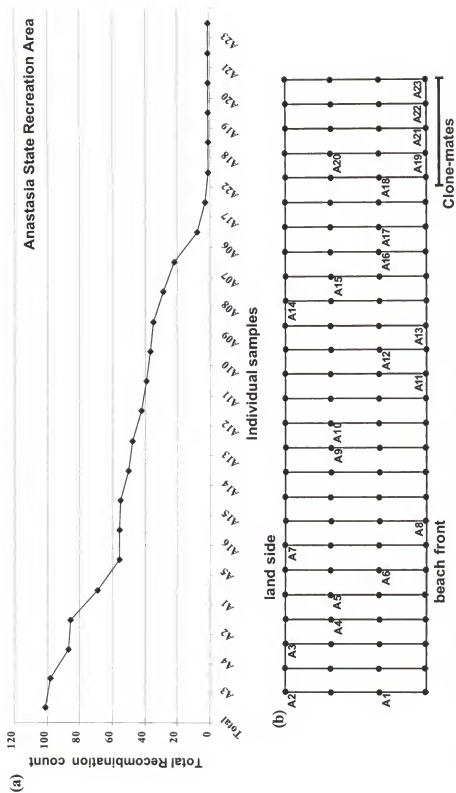


Figure 4.7. Successive deletion of sea-oats samples beginning with individuals having the highest individual recombination counts. a) Graphical representation of the reduction in total recombination counts against successive deletion of individuals from Anastasia and (b) the site plan showing spatial distribution of samples (A1 - A23) and clone-mates.

variability between individuals in each group. However, the maximum number of marker differences found between any two clone-mates was seven, while the differences were usually only two to three markers. The result of clonal reproduction is the stable transmission of genotypes across generations without the shuffling effects of genetic recombination (Avisé, 1994). Thus, the only source of variation between clone-mates is mutation (Avisé, 1994; Mes, 1998). Therefore, RAPD variations found between individuals with lack of recombination in each population are probably caused by acquisition of mutations during prolonged vegetative reproduction. However, the mutation rate seems to be high within these groups of individuals.

The occurrence of somatic mutations within clones has been well-documented using morphological (Hayward, 1972; Breese and Hayward, 1972), DNA sequence and molecular marker data in other plant species (Caetano-Anolles, 1998 and 1999; Primack and Kang, 1989; Silander, 1979 and 1985; Waycott, 1998;). Caetano-Anolles (1999) reported a genome-wide mutation rate of 10 nucleotide substitutions per generation within Bermudagrass cultivars using a molecular technique similar to RAPD, arbitrary signatures from amplification profiles (ASAP). In addition, an even higher level of genetic differentiation within clones of *Taraxacum* (Mes, unpublished data) has been observed compared to the differences we found in sea-oats. In some clonally reproducing species, both within- and between-population genetic variation exist at levels similar to or higher than levels found in outcrossing species; and somatic mutations may play a considerable role in maintaining variability in these natural populations (Ellstrand and Roose, 1987; Primack and Kang, 1989; see review Silander, 1985). Most of these mutations go unnoticed, because they have no apparent impact on the fitness of the

individuals (Silander, 1985). Many of the RAPD markers are selectively neutral and are expected to be more tolerant to selection (Williams et al., 1990).

The criterion of character incompatibility recognizes signatures in the fingerprint patterns that result either from asexual or sexual reproduction. In order to judge the impact of mutation, it is necessary to make assumptions regarding genetically identical plants as clone mates (or alternatively, genetically different plants as belonging to different clones). Excluding mutation amounts to assuming that the frequency of sexual reproduction exceeds the rate of mutation of the marker system that is used to document the genetic diversity (Brookfield, 1992). In practice, these assumptions can not be tested. If mutation is allowed, the assumption is that purely clonally evolving plants display a tree-like pattern of descent. The variability in fingerprint patterns of the compatible individuals with lack of recombination in sea oats populations suggest that the lack of signatures of sex is not due to a lack of variation. In addition, though the impact of mutation has mostly been a subject of the evolution of microsatellite loci (Schloetterer, 1997), it is related to the variability of a marker system. Because RAPDs and AFLPs are equally variable relative to microsatellites (Hulst et al., 1999), and because mutations have been found using RAPDs (somatic cells: e.g. Al-Zakin et al., 1999; Gill et al., 1999; germline cells: Lushai et al., 1997), mutation should be taken into consideration as a source of variation.

The genetic variation within clones due to mutation has not been taken into consideration in many genet-ramet determination studies and only identical individuals have been included in the analysis, due to the difficulty in obtaining accurate estimates of clone-mates (Ayers and Ryan, 1997; Graham et al., 1997; Gabrielsen and Brochmann,

1998; Sydes and Peakall, 1998). As a result, the number and spatial distribution of clone-mates existing in a population are probably frequently underestimated. Detailed knowledge of the number and distribution of clone-mates in populations is necessary to draw inferences such as: (1) sex ratios within mixed sexual-asexual populations; (2) magnitudes and patterns of gene flow; (3) mating systems and degrees of outcrossing; (4) extents of interclonal competition; and (5) the evolutionary ages of the populations (Avisé, 1994; Cook, 1985; Maddox et al., 1989).

Apart from the occurrence of clone-mate groups, all populations showed a limited spatial distribution of clone-mates except for EK, in which the clone-mates were widely distributed (Fig. 4.4b, 4.5b, 4.6b and 4.7b). A limited spatial distribution of clone-mates can be expected because of the expansion of clones only by rhizome development. Thus, these observations may indicate the extent of clonal spread of sea-oats by rhizome development. The spread of clones spatially by rhizomes may be a function of age and the fitness of the clones (Harper, 1985; Silander, 1985b). Apparently, the widely distributed clone found in EK population may provide information on either the age or the fitness of the clone or both. The dendrograms generated for the clone-mate groups showed further individual relationships within each group (Fig. 4.8). The position of individuals in each tree indicates the relationships among individuals of these clone-mates. Additional information on expansion of clones by rhizome development and mutational divergence may be useful for generating information on the age of sea-oats populations.

The possibility exists for production of closely related individuals by continued selfing in populations, although this is unlikely in wind-pollinated species (Lovell and

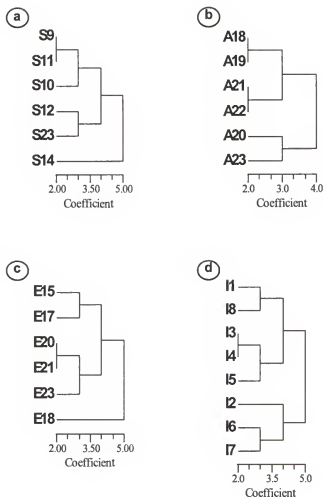


Figure 4.8. Dendrograms generated using Nei's minimum distances (Nei, 1972) for sea-oats samples belonging to clone-mate groups. (a) St. George Island, (b) Anastasia, (c) Egmont Key, and (d) Sebastian Inlet.

Hamrick, 1984). In Florida sea-oats, there is a possibility for off-season flowering (personal observation), which may enhance the possibilities for selfing due to the limited number of outside pollen donors. Although Crewz (1987) reported the possibilities for seed set within the same infructescence probably by selfing, further research is needed to test for self-compatibility in terms of seed viability, germination and seedling vigor. Hester and Mendelssohn (1987) reported extremely low sea-oats seed production in Caminada-Moreau coast populations, Louisiana. This has been attributed to very low outcrossing rates associated with the paucity of sea-oats plants in that area. Generally in grasses with wind-mediated pollination, progenies that result from selfing exhibit strong inbreeding depression (e.g., rye grass), which may hinder the persistence of purely self-pollinated groups of plants. Our study based on 101 RAPD markers generated high levels of polymorphism, however, without indications of sub-population differentiation between the adult plants (Ranamukhaarachchi et al., 1999). This is not a characteristic feature of populations resulting from selfing (Linhart and Grant, 1996). Given these possibilities, it is unlikely that sea-oats groups, similar to clone-mates, have resulted from self-pollination.

The major goal of this study was to evaluate the character incompatibility approach to determine the degree of recombination and detect clone-mates in sea-oats populations, which in turn will generate information on aspects of genetic diversity and reproduction modes. We found genetic variability differences among populations based on recombination counts that agree with the previous estimates of sea-oats genetic diversity in Atlantic and Gulf coast populations (Ranamukhaarachchi et al., 1999). We also found clone-mates in all four sea-oats populations. Thus, the incompatibility

approach may be useful to measure relative genetic diversity between populations and in particular, to generate estimates of clone-mates. This study is one of the first attempts to distinguish sexual from asexual populations and possibly to identify clones using the character incompatibility approach. Consequently, a comparison of incompatibility analysis results in this study with another study of similar nature is not possible. An advantage of this approach lies on the fact that DNA markers such as RAPD and AFLP generate a large number of polymorphic markers that facilitate the application of the character incompatibility approach to population studies.

CHAPTER 5

A MODIFIED AFLP TECHNIQUE FOR RAPID GENETIC CHARACTERIZATION IN PLANTS

Introduction

Molecular markers provide a useful tool to obtain a considerable fraction of the genetic information available within an organism (Awise, 1994; Karp et al., 1996; Jones et al., 1997). The selection of a molecular technique to generate markers depends on the specific objective, analytical power (resolution between samples), knowledge requirement, time, cost, and reliability. Restriction fragment length polymorphism (RFLP) markers generate accurate data and produce allelic information on different loci. However, resolution is low in terms of the polymorphism generated and applicability of RFLP is restricted due to the use of radioisotopes, the need for large quantities of DNA, and prior sequencing knowledge required to synthesize single-locus and multi-locus probes. With extensive allelic information, VNTR (variable number of tandem repeats also termed “microsatellites” and “minisatellites”) generate the maximum resolution between individuals, however, it is constrained by practical difficulties mentioned for RFLP as well as difficulty in generating microsatellite libraries. Both techniques generate co-dominant markers.

A number of techniques have evolved with the advent of polymerase chain reaction (PCR) technology such as random amplified polymorphic DNA (RAPD; Williams et al., 1990), arbitrarily primed-PCR (AP-PCR; Welsh and McClelland, 1990),

and DNA amplification fingerprints (DAF; Caetano-Annoles et al., 1991). These methods generate more markers than RFLP. Because of the large number of primer combinations that can be generated, PCR-based techniques theoretically provide an endless source of priming sites for thorough multi-loci screening of any organism. Techniques that utilize arbitrary primers are less expensive, rapid, do not require radioisotopes, and can be used to obtain genetic information on genetically unknown organisms. However, owing to low annealing temperature conditions, these techniques often generate artefactual bands associated with misannealing of primers and result in poor reproducibility of the markers. Intensity of the bands between individuals could be different causing ambiguity in scoring (Karp et al., 1996), especially when a large number of samples are used for the experiment. This has necessitated repeating the same experiment until a consistent banding pattern is obtained. This problem creates frustration due to waste of time and resources.

The more recently developed amplified fragment length polymorphism (AFLP) technique (Vos et. al., 1995) utilizes both principles of RFLP and techniques such as RAPDs. It generates an extensive level of polymorphism between individuals with high reproducibility and uniform intensity of bands. However, AFLP technique also requires radioisotopes, extensive laboratory skills and is time consuming. These limitations restrict using techniques such as AFLP to only more sophisticated laboratories (Weatherhead and Montgomerie, 1991). Progress in developing new techniques is needed in two directions, simplicity and analytical power (Hadrys et. al., 1992). Thus, an ideal technique would be one with the ease of RAPD while retaining the power of reproducibility and uniform intensity of bands generated between samples.

The standard AFLP technique consists of: 1) restriction-digestion of genomic DNA using two restriction enzymes (a rare cutter and a frequent cutter) in separate steps; 2) ligation of synthetically generated adaptors with DNA sequences corresponding to the sticky ends of restricted DNA products; 3) pre-selective PCR amplification using two primers complementary to the adaptor ligated ends with one pre-selected base at the 3' end; and 4) selective amplification generating the final products by a second series of radiolabelled primers generally containing three tandemly-oriented pre-selected bases at the 3' end. These amplified products are then separated by polyacrylamide gel electrophoresis (PAGE) on a DNA sequencing gel apparatus (Vos et al., 1995). Handling and disposal of radioisotopes and making sequencing gels require extreme care.

In this paper, we describe a modified AFLP protocol that was developed for rapid genetic characterization of plants. Major modifications to the standard AFLP technique involved: 1) using only one restriction enzyme, one adaptor, and one primer; 2) agarose gel electrophoresis to separate bands followed by ethidium bromide staining; and 3) incorporating formamide to generate more intense and uniform bands. This modified method involves considerably fewer steps compared to the standard AFLP technique (Vos et. al., 1995) while maintaining high reproducibility with analytical power equal to or greater than RAPD. Different modifications to AFLP have been reported for genetic characterization of fungi (Mueller et al., 1996), and honeybees (Suazo and Hall, 1999). Plants contain higher quantities of phenolic compounds and polysaccharides that may prevent complete digestion of DNA, which is an important step in AFLP technique. We have tested AFLP modifications for several plant species to determine 1) simplicity,

reproducibility, equal intensity, and analytical power, and 2) the ability to serve as a reliable molecular tool to generate markers in plants for wide range of applications.

Materials and Methods

Plant Materials

Four plant species, sea-oats (*Uniola paniculata* L.), pickerel-weed (*Pontederia cordata* L.), Bermudagrass (*Cynodon dactylon* L.), and *Penstemon heterophyllus* Lindl. were selected for the study. Sea-oats is a subtropical coastal dune grass, while pickerel weed is naturally found in wetland conditions. Bermudagrass is horticulturally important for turf management. *P. heterophyllus* is a self-pollinated, terrestrial, herbaceous plant. These species were selected to test the performance of AFLP modifications on generating polymorphism for different plant species. Ecotypic samples of pickerel-weed from Rhode Islands to South Florida, different cultivar samples of Bermudagrass (FHB-88, FHB-89, Floratex [BS.FB-119], FHB-285, FHB-319, Tifdwarf, Tifgreen, FHB-281) grown in eastern United States, samples from a disjunct population of *Penstemon*, and Florida population samples of sea-oats, were collected. DNA was extracted using a modified method of Murray and Thompson (1980), which uses CTAB extraction buffer. Modifications included, 1) replacing octanol by iso-amyl alcohol, and 2) precipitation of DNA by 100% ethanol.

Restriction Digestion and Ligation

Three restriction enzymes, *EcoRI*, *TaqI*, and *MseI* were used to prepare template DNA by single enzyme digestions. Adapters for ligation reactions were prepared as

reported by Janssen et al. (1996). The protocol for preparing template DNA is given here using *EcoRI*, and is common to all the restriction enzymes. Genomic DNA (0.5 µg) was digested using 10 units of *EcoRI* enzyme. At this stage, experiments were conducted to evaluate the quality of the final products as influenced by restriction digestion/ligation in one reaction and restriction digestion followed by ligation reaction in two steps. The latter was implemented based on the quality and the number of bands amplified. A total volume of 20 µl digestion mixture consisted of *EcoRI* (2 µl), 10X reaction buffer (2 µl), and a specific volume of genomic DNA solution that contained approximately 0.5 µg DNA. The digestion mixture was incubated at 37°C for 6 hrs., followed by the addition of DNA ligase (2 µl), 5X reaction buffer (2 µl), and 50 pmol *EcoRI* adopter (1 µl) to the same digestion mixture making a total volume of 25 µl. This mixture was incubated overnight (12 to 14 hrs.) at room temperature. Following digestion/ligation, template DNA was purified from excess adopters, enzymes, and other contaminants using 95% ethanol precipitation.

PCR Amplification and Gel Electrophoresis

In a series of preliminary experiments, PCR amplification conditions were optimized for Mg, *Taq* DNA polymerase, and template DNA concentrations. In another series of experiments using the above-optimized conditions, effect of dimethyl sulphoxide (DMSO), formamide, and bovine serum albumin (BSA) were also tested for improving DNA profiles. Based on these results, the final optimized amplification reaction conditions included 15 ng template DNA, 2.5 U *Taq* DNA polymerase, 40 pmol primer, 200 µM dNTPs, 2.5 mM MgCl₂, 2% formamide, and 1% BSA, 10 mM Tris-HCl

pH 8.5, 50 mM KCl, all in a total volume of 15 μ l. The optimized thermal cycling conditions were 2 min at 94°C followed by 40 cycles of 94°C/25 sec, 56°C/25 sec, 72°C/25 sec and final extension at 72°C/7 min. Primers were synthesized as described in Vos et al (1995). Each primer consisted of DNA sequences complementary to a major part of the adopter sequences containing a specific restriction site plus arbitrarily assigned bases to the 3' end of the primer. Two sets of primers were designed based on the number of arbitrary nucleotides used; two bases (18-mers), and three bases (19-mers).

PCR products were electrophoresed using a 'SynergelTM' and agarose (1.73 g; 1.05 g) gel mixture in 150 ml of 0.5X TPE buffer that would generate a similar strength to 3% standard agarose gel (Perlman, 1991; Diversified Biotech, Boston, MA 02132). Each lane consisted of 8 μ l of PCR products and the gels were run at 65 volts for 8 to 10 hrs. Bands were visualized by ethidium bromide staining (Sambrook et al., 1989).

Specific Performance and Reliability Tests

A number of experiments were conducted to test performance and reliability of the modified AFLP technique using *Eco*RI. These tests were: (1) performance of amplified products using two sets of primers with two and three unknown bases at the 3' end; (2) reproducibility of the final products, between two different restriction digestions and between PCR amplifications at different time periods (one, two, four, and 12 weeks); (3) the ability to generate adequate resolution power for a wide range of plant species which included cultivars, ecotypes, individuals within and between populations, and both self and cross pollinated plants; and (4) comparison of RAPD and modified AFLP methods for generating polymorphism for population studies.

Results and Discussion

AFLP Modifications and Optimizing Experimental Conditions

Of three restriction enzymes tested, based on the type of primer (18-mer or 19-mer) used, *EcoRI* and *MseI* generated amplified bands that could be separated by synergel:agarose gel electrophoresis. Both enzymes generated more than ten clearly visible bands when 19-mer primers having three selective bases were used, while, 18-mer primers with two selective bases caused a smear on each lane of the gel. This observation due to primer difference results from the reduction in selection of complementary DNA strands from 1/64 possibilities (1 in 4X4X4 nucleotides) in 19-mers to 1/16 (1 in 4X4 nucleotides) in 18-mers. Thus, when 18-mers are used, too many bands may be generated resulting in a smear. If 18-mers are used for amplification, it may be necessary to increase the resolution power of gels either by increasing synergel: agarose concentration or by using polyacryllamide gels for better separation of the bands. *TaqI* enzyme always produced bands with a smear in both cases.

Restriction digestion followed by ligation in two separate steps produced better results compared to digestion and ligation in one reaction. This is in contrast to the AFLP modifications reported earlier for other eukaryotic systems except plants (Mueller et al., 1996; Suazo and Hall, 1999). In general, plants contain higher quantities of polyphenolic compounds and polysaccharides that are often co-precipitated with DNA (Dellaporta et al., 1983; Rether et al., 1993). Both may lead to partial or poor digestion of DNA (Li et al., 1994; Karp et al., 1996), which is an important step in the AFLP technique. However, not combining the digestion/ligation into one step did not affect the rapidity and

efficiency of the technique because, after the digestion, we added the ligation mixture to the digestion solution (see material and methods) which is not a difficult step.

Addition of 2% formamide or 2% formamide plus 0.5% BSA improved DNA amplifications and band intensity. DMSO at 5% or 10% concentrations always produced no amplification. Specificity of bands was greatly enhanced at 2% level compared to no formamide allowing better band scoring. However, template DNA concentrations less than 10 ng/15 μ l of PCR mixture resulted in inhibition of amplification by formamide. Formamide weakens the hydrogen bonding between nucleotides and helps in destabilizing DNA in solution (Casey and Davidson, 1977; Zhang et al., 1991). Organic solvents also prevent primer secondary structure formation (Pomp and Medrano, 1991). These properties in organic solvents, by facilitating the primer annealing step, improve PCR in DNA sequencing and amplification, especially DNA regions with high GC contents (Zhang et al., 1991; Sarker et al., 1990; Winship, 1989; Pomp and Medrano, 1991). Therefore, adding formamide is a simple step useful in generating AFLP with improved intensity of bands.

Generating Polymorphism among Plant Samples

We screened eight AFLP primers (19-mers) that generated extensive polymorphism for all the species tested including ecotypic samples (Fig. 5.1a), cultivars (Fig. 5.1b), and individual samples within natural populations (Fig. 5.1c, 5.1d, and 5.1e). Of the plant samples tested, ecotypic samples of pickerel-weed and turfgrass cultivars (Guy and Li, unpublished data) have been previously tested using RAPD method. However, both required extensive random primer screening to generate adequate

polymorphism to allow separation of samples. For pickerel-weed and turfgrass cultivars, 40 and 60 random primers were screened, respectively. However, even after screening 60 RAPD primers it was not possible to differentiate some turfgrass cultivars (e.g. cultivar samples 'FHB-319', 'Tifdwarf', 'Tifgreen'). Using our modified AFLP technique, we were able to differentiate some of the cultivars that could not be differentiated by RAPD (e.g. 'FHB-319' vs. 'Tifdwarf' or 'Tifgreen') (Fig. 5.1b). However, we tested only four 19-mer primers in this case and, if more primers are used, there is a potential for further differentiating closely related individuals such as 'Tifdwarf' and 'Tifgreen'. This is a time saving and important application of modified AFLP technique over RAPD.

Among experiments conducted to determine the ability to differentiate between individuals, sea-oats generated high polymorphism for samples both within- and between-populations after screening only eight primers (19-mers) (Fig 5.1c). Similarly, highly polymorphic bands were observed for both pickerel-weed (Fig. 5.1d) and *Penstemon* (Fig. 5.1e) samples collected from single populations. Pickerel-weed is self incompatible (Ornduff, 1966), and, sea-oats exhibits outcrossing mediated by wind pollination (Hester and Mendelssohn, 1987; Crewz, 1987). However, *Penstemon* is an obligate self-pollinated species. AFLP technique generated extensive polymorphism in all three species at the individual level. All these species cover both wetland and terrestrial environments as well as plant taxa belonging to different families. These findings confirm that this modified AFLP technique can be conveniently used for genetic characterization of plants.

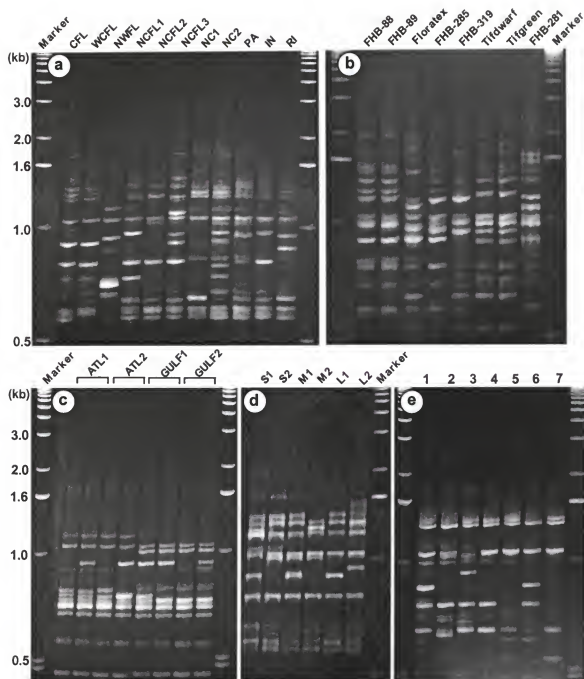


Figure 5.1. Polymorphism generated using a modified AFLP technique with four diverse plant species. a) Ecotypic samples of pickerel-weed (*Pontederia cordata* L.) collected in natural populations from Rhode Island to South Florida (samples listed by state abbreviations), b) Turfgrass cultivars of bermudagrass (*Cynodon dactylon* L.), c) Individual samples of sea-oats (*Uniola paniculata* L.) collected from two Gulf (GULF1 and GULF2) and two Atlantic (ATL1 and ATL2) coast populations in Florida, d) Individual samples of pickerel-weed collected from a single disjunct population in Gainesville, Florida, (S - Short, M - Medium, and L - Long morphological flower types), and e) Individual samples of *Penstemon heterophyllus* Lindl. collected from a single disjunct population in Gainesville, Florida. Notice that the modified AFLP procedure generated polymorphism in each species that can be used to identify individuals (within- and between-populations), cultivars, and ecotypic samples.

Reproducibility of Amplified Bands

For all the species tested, highly reproducible banding patterns were generated for both, PCR runs conducted over a period of one month (Fig. 5.2a) and different Digestion/ligation reactions conducted for the same DNA (Fig. 5.2b). To obtain a measure of reproducibility, we computed the total number of bands generated in each gel and the number of bands missed later from the initial amplification at different time intervals and digestions. The percent reproducibility of bands was then determined in each case. Between the two digestions and PCR runs, reproducibility was always maintained over 95%. We maintained high annealing temperatures, 56°C that prevented misannealing of primers and production of artifacts. This high reproducibility is a definite advantage of modified AFLP over other techniques that use low annealing temperatures and generate amplified bands with the same convenience of modified AFLP.

RAPD and Modified AFLP Comparison using Four Sea-Oats Populations

RAPD is considered as one of the most convenient and low cost techniques used in genetic characterization. A comparison between RAPD and modified AFLP was done to determine their analytical power. Six individuals each from four populations of sea-oats were used for the study. Eighty random primers were screened to select 10 that would generate polymorphic bands in RAPD. In contrast, only eight AFLP primers (19-mers) were screened and all generated considerable polymorphism (Fig. 3). Ten RAPD primers generated a total of 99 bands of which 49 were polymorphic. While, five AFLP 19-mers generated a total of 61 bands of which 27 were polymorphic. The average number of bands per primer was 9.9 in RAPD and 12.2 in AFLP, while the average

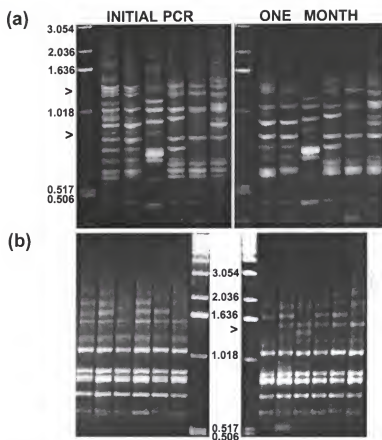


Figure 5.2. Reproducibility of amplified bands with the modified AFLP technique at two different levels; (a) reproducibility between PCR tests conducted at different time intervals for the same restriction digested/ligated template DNA samples, (b) reproducibility between PCR tests conducted for template DNA samples obtained by two different restriction digestion/ligation reactions for the same genomic DNA samples. > indicates the bands missing between PCR tests over a period of four weeks and two restriction digestion/ligations. Notice that the number of bands missing is very low.

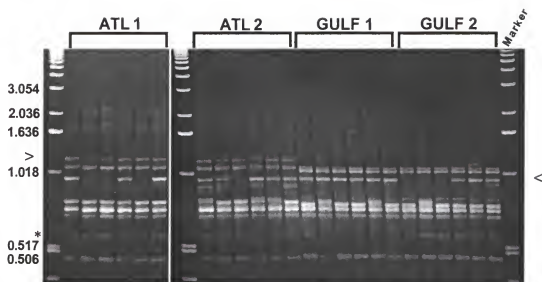


Figure 5.3. Amplified DNA profiles generated with the modified AFLP technique using samples from four sea-oats (*Uniola paniculata* L.) populations. Each population consisted of six individual samples. Of the eight primers (19-mer) screened, six generated polymorphism between samples of both within and between populations. a) DNA profiles for primer 5' GACTGCGTACCAATTCAAT 3'. > indicate polymorphic bands unique to a given coastal origin. * indicates separation of bands that differ by 11 bases at the 500 bp. range. ATL - Atlantic coast populations; Gulf - Gulf coast populations.

number of polymorphic bands per primer was 4.9 in RAPD and 5.4 in AFLP. As discussed before, the bands were more intense in AFLP compared to RAPD. However, the intensity of RAPD bands could not be improved by adding organic solvents. This characteristic feature of the modified AFLP facilitated the easy scoring of bands (as 0 and 1 data matrices) for statistical data analysis.

With the modified AFLP technique described above, two additional steps were necessary compared to other simple PCR-based techniques. These were, mixing genomic DNA with restriction digestion/ligation mixtures followed by purification of DNA in preparing the template DNA, which are not complicated steps. However, as observed in this study, time spent on preparing template DNA with the modified AFLP method is negligible compared to the time that can be saved on screening primers for generating polymorphic bands with high reproducibility. Other advantages of this technique and protocol are, improved uniform intensity of bands among individuals, high potential for screening genomes owing to the wide range of available restriction enzymes and the ability to design primer combinations.

CHAPTER 6

SUMMARY AND CONCLUSIONS

The prime objective of this dissertation study was to determine the population genetic structure of four Florida populations of Sea-oats, *Uniola paniculata* L., a pioneer coastal dune grass used for dune restoration projects. The main research goal was to determine the patterns of overall genetic variation both within and between sea-oats populations and to make inferences on the ecological and demographic determinants of resulting population genetic structure and discuss applicability of our findings for dune restoration. Novel approaches to generate and analyze molecular data were also implemented. Modifications to the standard amplified fragment length polymorphism (AFLP) method (Vos et al., 1995) were performed to develop a molecular technique that generates highly reproducible banding patterns with the same convenience of RAPD technique. A novel analytical approach, character incompatibility (Mes, 1998), was explored to obtain information on genetic recombination in sea-oats using RAPD data, which can then be used to detect reproduction modes and information on clones. A summary of the specific objectives, procedures, results and conclusions for all studies are outlined below.

**Molecular Analysis of Genetic Diversity in Four Florida Sea-oats
(*Uniola paniculata* L.: Poaceae) Populations: Implications for
Restoration of Dune Communities**

Adult plants and seedlings of four major sea-oats populations from both Atlantic and Gulf coasts in Florida were included in the study with the specific objectives to determine: 1) the level of genetic variation within sea-oats populations; 2) whether sea-oats exhibit significant genetic variation between populations; and 3) whether the populations on the Atlantic and Gulf coasts exhibit distinct genetic structure. The Atlantic populations were the more northerly-located Anastasia State Recreation Area ([AN], 29° 53' 15" N / 081° 17' 23" W) and east-centrally located Sebastian Inlet State Recreation Area ([SI], 27° 46' 42" N / 080° 29' 27" W). The Gulf coast populations consisted of a northern population from St. George Island State Park ([SG], 29° 39' 20" N / 084° 52' 53" W) and a west-central population from Egmont Key National Wildlife Refuge ([EK], 27° 35' 15" N / 082° 45' 45" W). Population sampling was done for both established adult plants and seedlings grown from seed (caryopsis) collected from the adult populations. Sampling included 23 adult plants and 18 seedlings from each of the four populations. Random amplified polymorphic DNA (RAPD) was used to generate molecular data using 10 random primers selected after screening 80. The resulting RAPD profiles were scored based on the presence and absence of bands and binary data matrices were developed for each population for further statistical analysis. Data were analyzed using average heterozygosity (H) (Hartl and Clark, 1997), Nei's coefficient of gene differentiation (G_{ST}) (Nei, 1987), F-statistics (Weir and Cockerham, 1984), gene flow (Nm) (Wright, 1951), and hierarchical analysis of molecular variance using both non-parametric (1000 permutations; Excoffier et al., 1992) and parametric (F-test; Weir, 1996) methods. These

analyses were based on certain assumptions such as homology of the equal molecular weight bands and the Hardy-Weinberg equilibrium status of the populations. Results were graphically represented using principle coordinate graphs and dendrograms based on Nei's minimum distance approach (Nei, 1972).

RAPD profiles generated a total of 101 and 109 bands for adult plants and seedlings, respectively. Based on the statistical analysis, very similar results were observed between the adult plants and seedlings for each population. Results indicated that a high degree of genetic variation was present both within and between all populations sampled. The Gulf coast populations showed a higher within population genetic variation compared to populations on the Atlantic coasts with the highest variation observed in the St. George samples.

Less genetic differentiation was observed between populations from the same coast compared to populations from both coasts. The least genetic differentiation was detected between the two Atlantic coast populations followed by two populations on the Gulf coast. Hierarchical analysis of genetic variation distribution, principle coordinate graphs, and the dendrograms indicated that populations from the two coasts were clearly separated. The two Atlantic populations were tightly clustered, while Gulf coast populations generated two proximal groups. Although similar genetic patterns were observed between adult plants and seedlings, seedling samples indicated higher genetic differentiation between populations, tighter clustering of Atlantic seedling samples, and more divergence within the Gulf coast than that of adult plants.

Possible ecological-based explanations for population-specific genetic variability and genetic divergence observed between coasts were discussed. High genetic

differentiation of populations between the coasts was attributed to the restricted levels of gene flow that prevented mixing of different genotypes resulted from different selection pressures on the two coasts. While, high levels of gene flow between populations on the same coast favored homogenization of populations. This difference in gene flow between populations may have been caused by the Florida peninsular barrier to movement of seed and pollen by wind between the two coasts. High within-population genetic differences on the Gulf coast were attributed to population disruptions associated with more frequent storm events on the Gulf coast leading to higher genetic variation by enhanced seed production and germination, enhanced seedling establishment, and restricted spread of genets by vegetative reproduction. Low genetic variation within Atlantic coast populations was attributed to the possibility of occurrence of few adaptable and dominating genotypes resulting from natural selection due to relatively more stable environmental conditions. Higher level of autogamy associated with the occurrence of vegetatively propagated widespread genets and as a result, inbreeding depression may have caused further lowering of genetic variation in Atlantic populations.

The significance of these findings for dune restoration was also discussed. Coastal differences in the genetic composition indicated that there could be ecotypic differences associated with the genetic variation. However, it was strongly suggested that these genetic differences be tested through common-garden and reciprocal planting studies because RAPD marker differences cannot be directly associated with the ecological adaptability of a given genotype. Based on the RAPD banding patterns, eight unique genotypes were selected from each of the adult sea-oats populations for micro-propagation and subsequent reciprocal transplanting studies. The occurrence of similar

genetic patterns between the adult plants and seedlings for each population indicated that the specific genetic variability characteristic of parent populations is maintained by sexual reproduction. If the maintenance of high genetic variability is a concern for dune restorations, this suggests that the use of seedlings may be the most suitable nursery material for planting, which is also the current practice in Florida.

Thus, the conclusions of this study were: 1) molecular analysis using RAPD revealed a high within- and between- population variation for both adult and seedling plants of sea-oats; 2) substantial genetic differentiation occur between the Gulf and Atlantic coasts; and 3) lower between-population variation was observed for populations on the same coast, with Atlantic coast populations being the least differentiated.

Character Incompatibility: an Approach to Determine Reproduction Mode of Sea-Oats (*Uniola Paniculata* L.) Populations

RAPD data generated from adult plants of sea-oats populations were used to determine the degree of genetic recombination using the character incompatibility approach. The major goals were to use genetic recombination, to obtain information on genetic variability differences within populations, and for identification and distribution of clone-mates. The principle behind this approach was that, two unordered binary characters with two states (e.g. 0/1 data), show recombination among individuals, if all four possible combinations are observed in the population (Mes, 1998). Thus the absence of recombination indicates the absence of sexual reproduction. Individuals that do not exhibit sexual reproduction probably represent individuals that belonged to the same clone (clone-mates). Recombination counts were obtained for entire population (total

recombination count) as well as for individuals (individual recombination counts) (Wilkinson, 1995), to obtain information on the relative levels of sexual and asexual reproduction. Clone-mates were obtained by successively removing individuals that have the highest recombination count, until the remaining individuals show very low or no recombination.

Total recombination count values were higher for the Gulf populations than the Atlantic populations. Especially, the recombination count for the SG population was significantly higher compared to other populations. Higher recombination counts on the Gulf coast were attributed to the presence of hybrid individuals that resulted from recombination of more diverged (polymorphic) parents. In particular, the SG population was comprised of genetically more divergent individuals. Successive deletion of samples with the highest individual recombination counts resulted in six to eight individuals per population with the lowest possible recombination count. This indicated the possible existence of clone-mates within each population. Although these individuals showed no recombination, genotypes were not identical except for two individuals in AN. These marker differences found between asexually-related individuals were attributed possibly to acquisition of somatic mutations with time. However, the maximum number of marker differences found between any two individuals was seven, while the differences were usually only two to three markers. The fact that sampling slots from which these clone-mates were correlated, were in close proximity to one another, suggests the occurrence of vegetative reproduction via rhizomes. Dendrograms generated for the clone-mate groups showed the relationships among individuals within each group of clone-mates.

In conclusion, character incompatibility study enabled: 1) determination of the degree of genetic exchange and relative levels of genetic diversity in sea-oats populations; 2) identification of clone-mates within each population; and 3) determination of the spatial distribution and genetic relationships among clone-mates.

A Modified AFLP Technique for Rapid Genetic Characterization in Plants

This study focused on developing a molecular technique that could be easily, but accurately, used in population studies dealing with large sample numbers. Although RAPD generates molecular markers more conveniently compared to other available molecular techniques, considerable experimental replication is usually necessary to detect reproducible banding patterns. To develop a highly reproducible and easy to apply molecular technique, a standard AFLP technique was modified and then compared with RAPD technique for superiority. The specific objectives were to determine: (1) the simplicity, reproducibility, equal intensity, and analytical power of modified AFLP method; and (2) the ability of the modified AFLP technique to serve as a reliable molecular tool to generate plant genetic markers for population studies and identification of individuals, cultivars, and ecotypes.

Modifications to the standard AFLP technique included: (1) using only one restriction enzyme, one adapter molecule for spliced sticky ends, and one primer; (2) agarose gel electrophoresis to separate bands followed by ethidium bromide staining; and (3) incorporating formamide to generate more intense and uniform bands. Four plant species, sea-oats (*Uniola paniculata* L.), pickerel weed (*Pontederia cordata* L.), Bermuda grass (*Cynodon dactylon* L.), and (*Penstemon heterophyllus* Lindl.) were used

to determine the ability of the technique to generate adequate resolution power. The study included examination of cultivars or ecotypes of these species, and individuals within and between populations in both self and cross pollinated plants. Reproducibility was tested at two major levels: 1) reproducibility between polymerase chain reaction (PCR) experiments for the same template DNA; and 2) between template DNA prepared by different restriction digested and ligated reactions.

The modified AFLP procedure generated highly reproducible bands in all the species tested. Of the organic solvents tested to improve PCR amplification, 2% formamide with or without bovine serum albumin (BSA) enhanced band intensities. Comparison between RAPD and modified AFLP using plants from four sea-oats populations showed the following advantages in modified AFLP procedure: 1) number of faint bands were greatly reduced with high specificity of amplified bands; 2) more number of polymorphic loci per primer could be detected; 3) primer screening time was less associated with a high percentage of primers producing polymorphic DNA profiles; 4) amplified products could be more easily scored; and 5) reproducibility of bands were greatly enhanced. These observations confirmed that this modified AFLP method can be easily used for genetic characterization purposes to generate markers that utilize multi-loci and dominant molecular data. In conclusion, this modified AFLP procedure provided a more convenient technique to generate molecular markers with high degree of reproducibility and specificity.

APPENDIX
AMPLIFIED DNA PROFILES OF
SEA-OATS (*UNIOLE PANTULATA* L.)

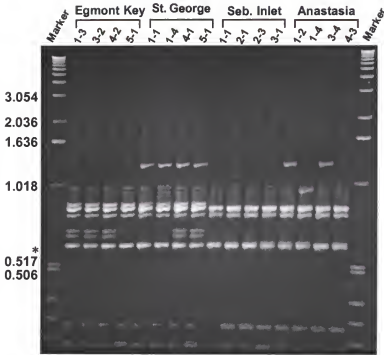


Figure A.1. PCR amplification profiles generated using random primer OPB 20 for DNA samples obtained from four different populations of sea oats in Florida. Gel electrophoresis was conducted using Synergel 1.72 g: Agarose 1.05 g mixture in a total volume of 150 ml of 0.5 X TPE Buffer which is equivalent to 3% standard agarose gel electrophoresis (Diversified Biotech). Gel was run for 8 hrs. at 65 volts. * indicates separation of bands differ by 11 bases at the 500 bp. range.

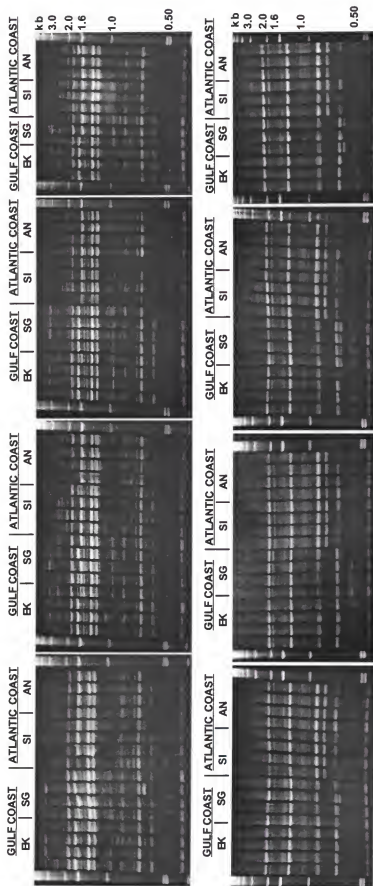


Figure A.2. PCR amplification profiles generated using random primers (a) OPL9 and (b) OPL18 for 92 DNA samples obtained from four sea-oats (*Uniola paniculata* L.) seedling populations in Florida. Each RAPD profile consists of a set of samples that are not repeated in other profiles. EK - Egmont Key, SG - St. George, SI - Sebastian Inlet, AN - Anastasia.

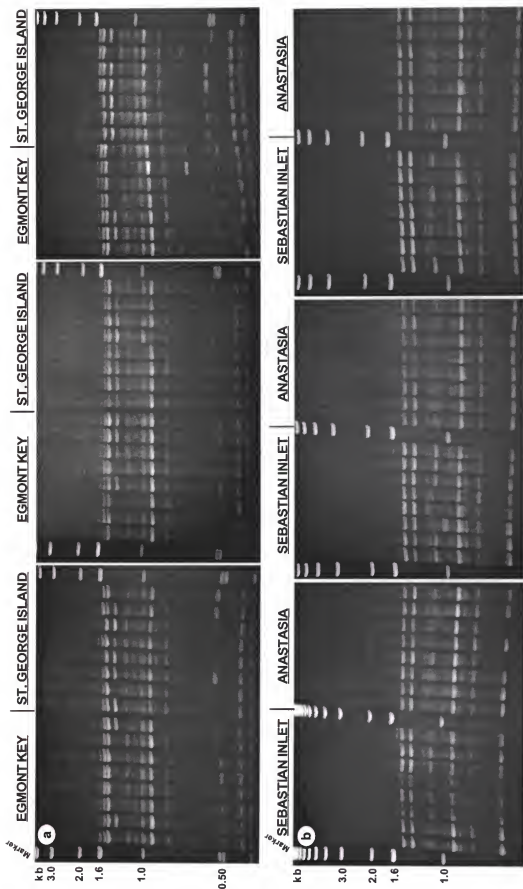


Figure A.3. PCR amplification profiles generated using random primers OPAJ2 for 92 DNA samples obtained from adult plants of four sea-oats (*Uniola paniculata* L.) populations in Florida. (a) two Gulf coast populations, Egmont Key and St. George Island, and (b) two Atlantic populations, Sebastian Inlet and Anastasia. Each RAPD profile consists of a set of samples that are not repeated in other profiles.

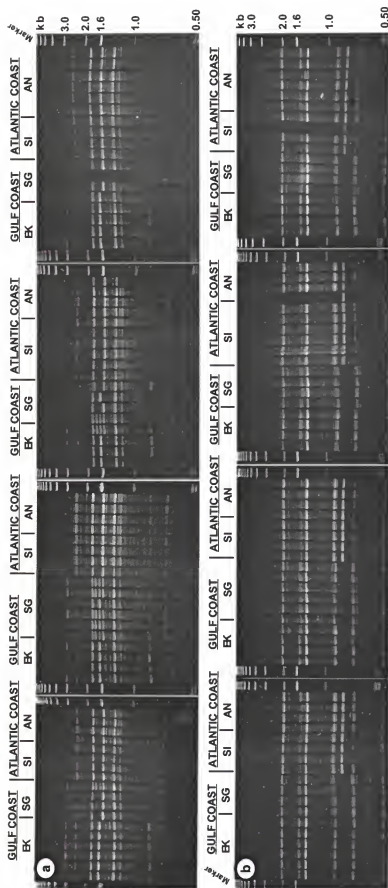


Figure A.4. PCR amplification profiles generated using random primers a) OPL 9 and b) OPL 18 for 72 DNA samples obtained from four sea-oats (*Umiola paniculata* L.) seedling populations in Florida. Each RAPD profile consists of a set of samples that are not repeated in other profiles. EK - Egmont Key, SG - St. George, SI - Sebastian Inlet, AN - Anastasia.

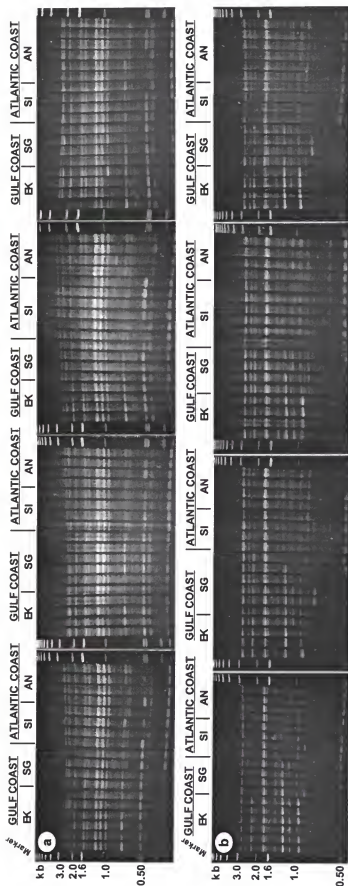


Figure A.5. PCR amplification profiles generated using random primers a) OPAK6 and b) OPAJ4 for 72 DNA samples obtained from four sea-oats (*Uniola paniculata* L.) seedling populations in Florida. Each RAPD profile consists of a set of samples that are not repeated in other profiles. EK - Egmont Key, SG - St. George, SI - Sebastian Inlet, AN - Anastasia.

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BIOGRAPHICAL SKETCH

Dayawansa Gunathilake Ranamukhaarachchi was born in Agalawatte, Sri Lanka. He completed his high school education at Nalanda Vidyalaya, Colombo, in August 1981 and entered the University of Peradeniya, Sri Lanka, for his bachelor's degree. In August 1986, he received his Bachelor of Science degree with honors in agriculture. In July 1988, Daya Ranamukhaarachchi joined the Sri Lanka Department of Agriculture and obtained research experience in the fields of plant breeding and tissue culture until June 1994. During this period he participated in various international as well as in-country training programs. In June 1994, he entered the University of Florida to pursue a Master of Science degree in horticulture. Upon completion of the master's degree in August 1996, he pursued doctoral studies in environmental horticulture at the University of Florida, to determine the molecular population genetic aspects of Florida sea-oats, an important dune-grass used to prevent coastal erosion. He will be working at the Centers for Disease Control in Atlanta, Georgia, as a postdoctoral research fellow following his doctoral degree.

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Michael E. Kane, Chair

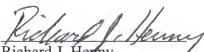
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Professor of Horticultural Science

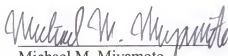
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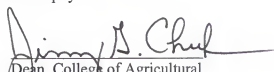
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Michael M. Miyamoto
Professor of Zoology

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 2000


Dean, College of Agricultural
and Life Sciences

Dean, Graduate School